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VIEWPOINTS IN BIOLOGY

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1

Edited by

J. D. CARTHY
Department of Zoology, Queen Mary College London

and

C. L. DUDDINGTON
Department of Chemistry and Biology, The Polytechnic Regent Street, London

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INTRODUCTION

Viewpoints in Biology will publish broadly based reviews of biological subjects which may be extensively illustrated. These reviews should not only summarize the state of the subject but also indicate the direction in which progress may be expected, and stress unsolved problems. While putting a cogent, well-argued point of view the authors will, however, not necessarily be asked to give exhaustive documentations of all the work in the subject. Considerations of the theoretical aspects of biology, criticisms of well-established methods and discussions of material from an evolutionary point of view will all be welcome. Descriptions of the practical details of methods will not be accepted unless they are involved in the consideration of a subject of general biological interest.

As far as possible the reviews should be readily understandable to other scientists as well as biologists. With increasing specialization within biology as well as the sundering of science into departments, it is more and more necessary that the problems facing one sort of scientist should be presented in a way which is understandable to others, so that if their interests are aroused they can bring to bear their own specializations on to the problems of another department of science.

Contributions will be printed in English but may be submitted in French or German.

Suggestions for contributions should, in the first place, be sent in outline form to one of the editors:

J. D. Carthy, Department of Zoology, Queen Mary College,
Mile End Road, London, E.1.

C. L. Duddington, Department of Chemistry and Biology,
The Polytechnic, Regent Street, London, W.1.

A guide to authors and information on fees will be sent when a suggestion is acceptable.

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CONTENTS

1

THE PHYSIOLOGY OF ROOTS

H. E. STREET

INTRODUCTION

TEXTBOOKS relating to any particular branch of science tend to follow a traditional pattern. The chapter headings of a new textbook dealing with the physiology of plants can usually be quite accurately and quite properly predicted from a study of the classical texts. It is, however, an equally well established tradition for the teacher to endeavour to present his subject in one way and to choose as collateral reading for the student a book which follows as contrasted a pattern as possible. Further, to test the student's progress the teacher seeks to frame questions which do not correspond to the sub-headings of either his lectures or the recommended textbook. These practices follow from the thesis that it is the ability to see interrelationships between different aspects of a subject which is the hallmark of understanding.

A heading similar to the one chosen for this chapter will not readily be found in any textbook. While it does discuss some work only recently published or still unpublished, its framework comes not from such new knowledge but from an attempt to bring together, from a number of aspects of botanical enquiry, information regarding the physiology of the roots of seed plants. By so doing, familiar information may gain new interest and significance. Further, such an approach, by its attempt to be comprehensive, high-lights some of the important gaps in our understanding of the physiology of higher plants.

The root system clearly serves to anchor the plant in the soil, frequently acts as a site for food storage and normally, in land plants, functions as the organ mainly responsible for the absorption of water and essential mineral salts. These physiological functions are reflected in the general form and microscopic structure of roots. Studies of size and form lead to studies of the rate, duration, localization and control of growth in the developing root. Studies of microscopic structure, by revealing the characteristic pattern of tissues and the wide range of different cells present, lead us to enquire into the factors controlling cell differentiation and into the separate contributions and physiological interrelationships of the individual cells and tissues to the vital activities of the whole root. Man can conserve or dissipate the fertility of the soil: the aerial environment of the plant is less within his control. Hence, every development in our understanding of the influence of the nutritive and other soil factors on root growth and function is potentially of importance in plant husbandry.

THE ROOT AS AN ABSORBING SYSTEM

Absorption of Water

Appreciation of the great extent and rapidity of growth in root systems followed from work on the prairie plants of the U.S.A. by Weaver and Clements[1] at the Carnegie Institution of Washington, and from the work of Rogers[2] at East Malling, on the growth of fruit tree roots. Examples may serve to illustrate this point. A two-year-old plant of the grass, *Agropyron cristatum* has been reported[3] to possess 315 miles of root, occupying a soil volume of 2 m by 1·2 m: a plant of winter rye, grown for 4 months in soil, was reported[4] to possess 387 miles of roots corresponding to an average production of 3·1 miles of root per day. In this latter case it was calculated that the new root produced each day invaded sufficient soil to provide for the total water requirements of the plant. Where, as must often be the case, the water content of the soil is such that only very slow movement of soil water occurs, then the invasion of new soil by root growth appears to be critically important in maintaining the water balance of plants. The very

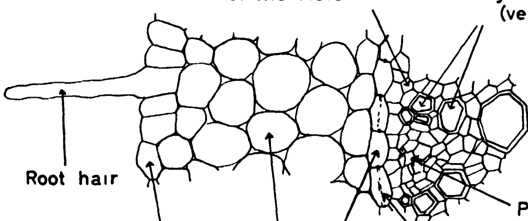


Figure 1.1. Diagrammatic transverse section of a root in the region of the root hairs

large quantities of water lost by plants on sunny days would require the root systems to have an enormous area of contact with the soil; for instance, a tree 10 m high may lose 200 l. of water per day by transpiration.

The uptake of water does not occur uniformly over the whole root surface. The most active region of water absorption is behind both the apical centre of cell division (the promeristem) and the region of most active cell elongation; it declines again as the outer layers of the root are rendered less permeable by the deposition of fatty material (suberin). This means that the most active region of water absorption from the soil is the region of root hair development, a region where the area of contact with soil particles is highest per unit length of root. The importance of this enhanced area of contact between soil particles and absorbing cells is a further indication of the slow movement of water in soils containing less than their maximum water content under conditions of free drainage (*i.e.* less than their field capacity). Studies of the development of root hairs in a moist atmosphere may give an exaggerated picture of the extent to which they enhance root

surface area in the soil. Nevertheless, root hairs on actively growing root systems in the soil probably increase root surface area by a factor of 2 to 5. We know that the functional life of root hairs is limited, that it may be as short as a few days. There is, however, little precise information regarding the natural variation in the duration of function of root hairs as between species and as affected by soil conditions. Nevertheless, it is clear that the role of root hair development in determining the absorbing capacity will only be of importance in actively growing roots. Rosene's work[5] at the University of Texas indicates that in seedling roots, the root hairs have only a

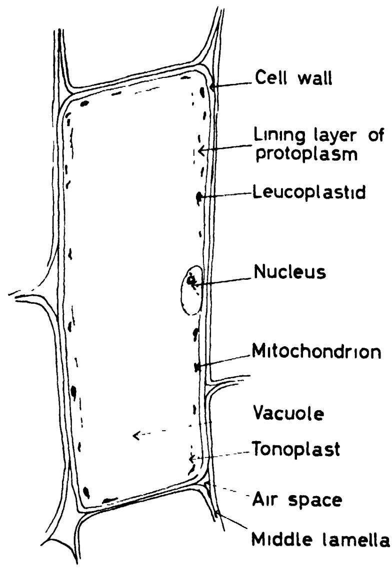


Figure 1.2. Diagrammatic section of a mature cortical cell of the root

similar permeability to water to that of other surface cells of the root. Examination of the roots of trees strongly indicates that water absorption by regions free of root hairs and even by suberized regions must, in many cases, account for the greater part of the water uptake. The mechanism of water uptake by suberized roots is at present obscure and in need of critical study.

The process of water uptake by the root involves not only the initial absorption but the distribution of water to the internal tissues. It is, therefore, necessary to consider the structure of the root—the nature and arrangement of cells in the root tissues. *Figure 1.1* is a diagrammatic representation of the transverse section of a root in the region of the root hairs. The cells of the piliferous layer, including the root hair cells, those of the cortex, including its special innermost layer, the endodermis, the parenchymatous cells of the stele and the phloem cells are living units. The living contents (protoplasts) have in the piliferous layer and cortex a large central aqueous vacuole separated from the enclosing wall by a sheath of protoplasm (*Figure 1.2*). The xylem cells (vessels or tracheids) when fully mature are represented

only by their cell walls. The root hair and piliferous layer cells have, like those of the parenchyma of cortex and stele, walls of cellulose and pectic substances which exert a strong attraction for water. The outer part of the walls of root hairs is slimy and strongly basophilic. This layer, particularly near the tip of the root hair, is permeated by a 'fuzz' of cellulose strands (as seen by electron microscopy) which develop intimate contact with soil particles. The living tissue of the cortex always has an intercellular air space system, the extent of which is influenced by the soil environment. This system only floods under conditions unfavourable to root growth, suggesting that the wall surfaces lining the air spaces are water repellent. The central stele, which functions in the longitudinal transport of water and solutes, is surrounded by a single continuous layer of cells constituting the endodermis. The radial and transverse walls of its cells, very early in their development, show evidence of the deposition within them of fatty material (possibly suberin). This constitutes the Casparian strip, first studied by Caspary in 1858 and readily revealed by its characteristic staining reactions. Fluorescein and other soluble materials capable of diffusing rapidly in the cell walls of the external cortex do not penetrate beyond this Casparian strip. Priestley[11] and others have stressed the importance, in the water and salt relations of roots, of the development of this Casparian strip on the grounds that it ensures that substances passing in either direction across the endodermis must pass via the living protoplasts of the cells.

It has long been known that the longitudinal movement of water in the root, and from the root into the shoot system takes place in the xylem cells, the vessels and tracheids. However, the state of this water in the xylem cells is dependent upon environmental conditions. Under conditions favourable to 'metabolic activity' but in which the rate of loss of water from the shoot by evaporation is very low, the water in the xylem cells is under positive pressure. This pressure can be demonstrated as a positive 'root pressure' by cutting off the shoot at ground level and attaching a manometer to the exposed transverse section of the root—an experiment performed by Evelyn in 1670 and by Hales in 1727. By contrast, under conditions of rapid water loss by evaporation from the shoot (*i.e.* with rapid transpiration) there develops a negative pressure in the xylem cells; the xylem water is under tension by virtue of a 'transpiration pull'. In the first case, the motive force of water movement is generated by the living root; in the second case, the water movement is a 'transpiration stream'.

The development of positive root pressure and the associated phenomenon of the 'guttation' of liquid drops of water from leaves are clearly the result of an 'active' absorption of water by the living root. Such root pressures frequently exceed 1 atm and White[6] at the Rockefeller Institute, using tomato root cultures, has recorded a root pressure of 6 to 7 atm. These positive root pressures develop only when there is a favourable temperature and adequate aeration, and their magnitude is affected by the ionic strength of the soil or nutrient solution in which the plant is growing. This 'active' water absorption is not capable of extracting from soil more than half the water removable under conditions of active transpiration. It may be of importance in submerged aquatic plants as a means of generating a flow of water and dissolved substances in the xylem cells. Kramer[7] of Duke

University has, however, argued strongly that it is of negligible importance in the over-all water relations of land plants, pointing out that 'active' absorption, under the most favourable conditions, only transports less than 5 per cent of the water moved by the actively transpiring plant and that positive root pressures can only rarely be demonstrated in conifers.

The mechanism leading to the generation of root pressure is a matter of dispute and to understand the problems involved it is necessary to refer back to the cellular structure of the root. There is strong evidence, from microscopy and from studying the penetration of dyes into cells, that a protoplasmic membrane (the tonoplast) is present at the surface of the cell vacuole. Whether a cell membrane also occurs at the protoplasm--cell wall junction is more difficult to determine, particularly as normally the protoplasm not only penetrates the cell wall as microscopically visible strands connecting the protoplasts of adjacent cells, but also may permeate the cellulose framework of the whole wall. Now ever since the classical researches of Hugo de Vries in 1871, we have known that the vacuolated plant cell can behave, under certain experimental conditions, as an osmotic system. The direction of, and force involved in water movement between the cell and its bathing solution depend upon the difference (called the suction force) between the osmotic attraction of the vacuolar solution tending to draw water into the cell and the sum of the external osmotic attraction of the bathing solution plus the inward pressure of the stretched cell wall (wall or turgor pressure), both of which tend to drive water out of the cell. When the water balance in the cell is determined by this relationship, the vacuole and the bathing solution lose or gain water, respectively, as if separated by a membrane permitting passage of water molecules but not of dissolved substances. Such a membrane was termed by Moritz Traube in 1867 a 'semipermeable' membrane. The operative cellular membrane is not the cellulose–pectin cell wall, which is readily permeable to solutes, but must be a protoplasmic membrane and, as indicated in the above description, it is generally considered to be the tonoplast membrane bounding the vacuole which confers upon the cell its osmotic behaviour.

On rather inadequate evidence, the view is widely held that, during 'active' absorption of water by the root, water passes from the soil or nutrient solution into the root hairs and piliferous layer cells and across the cortex by a gradient of inwardly increasing osmotic attraction (suction force). On this view, it is visualized that, by osmosis, water passes through the outer cell wall, the adjacent layer of protoplasm, into the vacuole, then from the vacuole via the protoplasm into the inner wall and so on through each of the cortical cell layers to the endodermis. However, studies[8,9] on the transport of salts and fluorescent dyes have provided evidence that solutes can move across the root without entering the vacuoles of the cortical cells and it has been calculated[10] that this 'apparent free space' in which solutes move freely significantly exceeds the cell wall volume in the wheat root. Water movement across the cortex may therefore occur along a pathway which includes the cell walls, films of water on the walls and the lining layers of protoplasm of the cells, but which does not involve penetration of the vacuolar membranes.

Priestley[11] and his co-workers first stressed the importance of the

endodermis in the development of positive root pressure and this has been supported by many later studies. Certainly, solutes which readily migrate in cellulose cell walls do not pass the Casparian strip. Furthermore, the cell wall in the region of the Casparian strip seems to be in particularly intimate contact with the protoplasm of the endodermal cells. The endodermis, therefore, may be the layer at which water (and solute) movement comes under protoplasmic control. This view is not universally accepted for evidence has been obtained[12] of rapid movement of solutes across the endodermis even when, as in the older parts of the root, its protoplasts become enclosed in layers of suberin.

The fact that positive root pressure is only observed under conditions promoting the physiological activity of the living root clearly points to a protoplasmic control of the underlying 'active' water uptake, but it does not indicate what aspect of the metabolism of the cells is most directly linked to this process. Priestley put forward the hypothesis that osmotically active substances arise in the xylem cells during their maturation, when disintegration of their protoplasts occurs, and that the living endodermis acts as the effective semipermeable barrier between the osmotic attraction of the liquid in the xylem and the soil solution. Atkins[13] visualized an osmotic movement of water right through the living tissues of the root dependent upon the difference in osmotic attraction of the soil solution and the xylem liquid, postulating a secretion of sugars into the xylem by the living cells of the stele. However, more recent work suggests that inorganic salts, liberated by the living cells of the stele, are mainly responsible for the osmotic attraction of the xylem liquid. The contention in these hypotheses, that root pressure is a consequence of a difference in osmotic attraction between the xylem liquid and the soil solution, has remained a widely accepted view and in support of this Kramer[7], for instance, has demonstrated that root pressure changes extremely rapidly in response to any variation in the osmotic activity of the solution around the root, just as if the root were acting as a sensitive osmometer.

The classical hypothesis that osmosis is the force regulating the water content of plant cells has already been outlined. However, of recent years there have been reported a number of instances where the hydrostatic pressure established in cell vacuoles appeared to be in excess of that expected from osmotic considerations. This has led to the postulation of a 'non-osmotic' force in water uptake, dependent upon the continuous expenditure of energy by the cell. If the water balance of cells is, in fact, regulated entirely or even in part by such a 'pumping' force, then it could move water into or out of cells. This is the basis of the alternative concept that root pressure is not the reflection of an osmotic system but results from an active secretion of water into the xylem cells. However, experiments using isotopically labelled water (tritium-labelled and deuterium-labelled water) show that cells are very permeable to water. Therefore, it seems unlikely that a pumping or secretory mechanism could operate against so 'leaky' a membrane. Nevertheless, it is still necessary to decide whether the 'active' component in root pressure is a secretion into the xylem cells, of solutes or of water. Further, we have little knowledge of the mechanism by which cells exhibit such secretory activity.

This consideration of active water absorption by the root has been dictated by its physiological interest rather than because of its importance in the water relations of the whole plant. As indicated earlier, the water in the xylem cells is normally under negative rather than positive pressure due to the development in the leaves of a transpiration pull. Actively transpiring plants can lower the water content of the soil to a point where it has a water absorbing power of, on an average, 15 atm; 'active' root absorption ceases when this water absorbing power of the soil is of the order of only 1 to 2 atm. From studies of the rate of intake of water by root systems attached to a vacuum pump it has been shown that resistance to flow falls when the root is killed, indicating destruction of protoplasmic regulation of flow and that the rates of water absorption achieved under active transpiration would indicate tensions of 5 to 30 atm in the xylem cells. Such negative pressure in the xylem cells opposes the attractions of the root cell walls and protoplasts for water and causes a flow of water from the soil to the conducting xylem cells. The extent to which this will reduce the water content of the root cells will depend not only upon the magnitude of the tension in the water columns in the xylem but also upon the water content of the soil and the resistance to water flow offered by the root tissues. The main resistance to water loss by transpiration is at the conversion of liquid water into water vapour within the leaves. Withdrawal of water from the root cell protoplasts to the point where there is no positive hydrostatic pressure within the root cells (to the point where the cells are no longer turgid) will, therefore, presumably only occur when the water absorbing power exerted by the soil begins to reach the critical value averaging 15 atm. The rate of water uptake thus seems normally to be determined by the rate of transpiration, the root providing an ever-expanding but passive absorbing surface.

Absorption of Mineral Ions

Many early botanists assumed that because mineral salts occur in the soil solution they must be absorbed with the water. This question of what, if any, is the relationship in roots between the processes of water and salt absorption is still unresolved. As will be illustrated in the ensuing account, root cells can selectively accumulate mineral ions. In such accumulation there is no sweeping along of mineral ions in a flow of water. It has, however, been suggested that the increased rates of salt uptake frequently recorded during periods of high transpiration rate may arise by a direct flow of soil solution via the 'apparent free space' into the xylem cells. However, this would seem to ignore the evidence for a controlling function of the endodermis. Alternatively, the increased rates of salt uptake associated with periods of rapid water uptake could be due to prevention of local depletion of particular ions at the site of their active absorption or to enhanced secretion of salts into the xylem cells associated with the greater water movement.

The soil solution is normally extremely dilute when considered in relation to the mineral ion requirements of plants. Great interest therefore attaches to evidence that roots can absorb basic ions (cations) directly from colloidal particles. Two hypotheses have been advanced to explain this uptake from

soil particles of cations such as calcium, potassium, and magnesium. The first stresses that roots constantly release carbon dioxide during their respiration, this in turn forms carbonic acid and leads to an exchange of hydrogen ions for cations. Acid production by soil micro-organisms could effect a similar release. The released cations would be absorbed along with bicarbonate or other acidic ions (anions). The second hypothesis postulates a 'contact exchange' of ions whenever the colloidal root surface makes contact with the soil colloids. This exchange proceeds without the ions ever appearing in the soil solution. As regards the source of essential anions such as phosphate, nitrate and sulphate, the general view seems to be that they are absorbed only from the soil solution and that the plant does not directly influence the release of these ions from the solid phase of the soil. A constant release of such anions from insoluble solids or from microbial activity may, therefore, be critical for plant growth. Further consideration of the soil–root relationship along these lines would take us away from our main theme into the many-sided problems of soil fertility.

Intensive studies of salt absorption by roots, grown under standard conditions and detached from the shoot, were undertaken during the 1930's by Hoagland[14] and his associates in the Division of Plant Nutrition of the University of California (Berkeley) and by Lundegårdh[15] and his associates at the Royal Swedish College of Agriculture. In these researches the influence of concentration upon the uptake of different ions was studied. Different ions were found to differ in their rates of uptake, ions with a low charge density being usually particularly rapidly absorbed. Cations modified the rate of uptake of other cations, anions the uptake of other anions. Changes in reaction of the external solution towards acidity or alkalinity were found to be indicative of unequal rates of absorption of cations and anions. All such observations emphasized that ion accumulation was a selective process and that it involved movement of ions into cells against concentration gradients, often resulting in the building up of high internal ion concentrations from very dilute solutions. Perhaps even more important, these researches, confirming parallel studies undertaken with the algae *Nitella* and *Valonia* and with storage tissues, emphasized the importance of the physiological activity of the cells in determining their ability to effect salt accumulation. Ionic balance within cells was maintained despite the unequal uptake of cations and anions, by the physiological production or depletion of organic acids. Respiration of the root cells was essential and the accumulation process ceased in the presence of substances which inhibited respiration or which 'uncoupled' this process from the chemical reactions which lead to the conservation of the released energy in a utilizable form. This relationship was of particular interest, for not only was respiration essential to salt accumulation but whenever accumulation was proceeding the rate of respiration was itself enhanced. This led Lundegårdh to postulate that the total respiration of cells is made up of two components, a 'ground' respiration independent of salt accumulation and a 'salt' respiration.

Experiments in which cells are allowed to take up radioactive ions and are then placed in a nutrient solution containing the normal ions have demonstrated that ion exchange between the cell and its bathing solution takes place more or less rapidly and continuously. Therefore, presumably not

only the initial accumulation process but also the maintenance of the excess internal concentrations of ions requires the expenditure of energy. Now one of the principal factors controlling respiration rate is the availability of energy-acceptor molecules, that is, of molecules capable of energy enrichment, such as adenosine diphosphate which can be energy-enriched by further phosphorylation. Presumably, salt accumulation, by consuming cellular reserves of utilizable energy, will generate such energy-acceptor molecules and hence stimulate respiration.

These researches, therefore, pose two fundamental and clearly intimately related questions regarding salt accumulation by plant cells:

(*a*) How exactly is the energy released by respiration used to transport ions against their concentration gradients?

(*b*) What determines the selective nature of the ion uptake mechanism, a selectivity which varies according to both the inherited potentialities and physiological state of the cells?

In a typical cortical cell of the root, the lining layer of protoplasm contains the single nucleus and numerous smaller living inclusions, mitochondria (*Figure 1.2*). Each of these bodies and the vacuole is enclosed in its own membrane. The outer surface of the protoplast is negatively charged but, as previously discussed, the extent to which it is bounded by a membrane is controversial. Because of this negative charge at the protoplast surface and on the colloidal particles of the protoplasm, it might be expected that the positively charged cations would penetrate the protoplasm of the cell more readily and to a greater extent than would negatively charged anions, and there is a considerable body of evidence that this is so. In this sense, ions may penetrate into cells by diffusion and the amounts so entering could be considerable if the penetrating ions were incorporated into various chemical compounds by physiological activity—nitrates into organic nitrogen compounds such as amino acids, phosphates into sugar esters, metal ions into combinations with protein, and so on. Active accumulation, that is, an accumulation which itself requires energy, would, however, be involved in the passage of cations into vacuoles and mitochondria, and in the case of anions would probably be concerned in their accumulation in the lining layer of protoplasm.

In order to explain such 'active' accumulation and its selectivity the idea has been advanced by many workers[16] that membranes which are impermeable to free ions are penetrated by the ions chemically combined with or adsorbed on to 'carrier' molecules located at the membranes (*Figure 1.3*). These carrier–ion complexes would then liberate their ions on the cell or cellular inclusion side of the membrane much more rapidly than the free ions can diffuse back along the concentration gradient. Selectivity would be determined, at any given time, by the relative frequencies of different 'carriers' in the cell membranes and by their individual mobilities and chemical affinities. Cell membranes are rich in phosphatides and proteins and one obvious suggestion is that the 'carriers' might be specific enzyme proteins, forming unstable enzyme(=carrier)–substrate(=ion) complexes. This interpretation is emphasized in the general term 'permeases' for solute carriers adopted by Monod and his associates at the Pasteur Institute of Paris. Using as a basis the assumption that the carrier–ion complex is

equivalent to the enzyme–substrate compound, kinetic analysis requires that the reciprocal of the velocity of absorption of an ion ($1/v$) should, when plotted graphically against the reciprocal of the ion concentration ($1/s$), give a straight line of which the ordinate intercept is at the reciprocal of the maximum rate of uptake ($1/V$) and the slope is given by K_s/V where K_s is the Michaelis constant (*Figure 1.4*). Epstein[17] and his associates have studied the uptake of ions by detached barley roots from simple and mixed salt solutions and reported results consistent with the hypothesis, set out above, that ion uptake involves the formation and breakdown of ion–carrier complexes and indicative of the functioning of separate carriers for sodium, potassium, calcium, magnesium, sulphate and phosphate ions. In the latter case, separate carrier sites seem to be involved for uni- and bivalent phosphate ions.

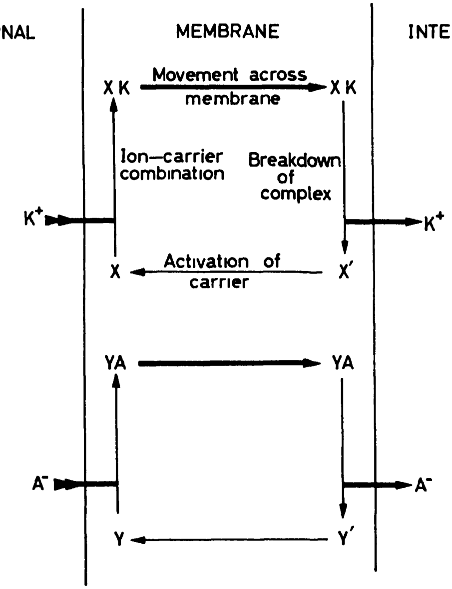


Figure 1.3. The general concept of the operation of a carrier mechanism in ion uptake by cells
X and Y = carriers. X′ and Y′ = precursors of the carriers.
XK and AY = carrier–ion complexes. K^+ = cation, A = anion.

It must be emphasized that the isolation and identification of the postulated carriers has not been achieved but modern biochemical techniques should make such isolations possible in the near future. A brief outline of two interesting suggestions will, however, illustrate how the carrier mechanism could be energy-requiring. Bennet-Clark[18] has suggested that the carrier could be a protein associated with the phosphatide, lecithin. The phosphate group in the phosphatide would act as a cation carrier, the basic choline group as an anion carrier. The liberation of the ions into the cell could be effected at the inner surface of the membrane by an enzymic

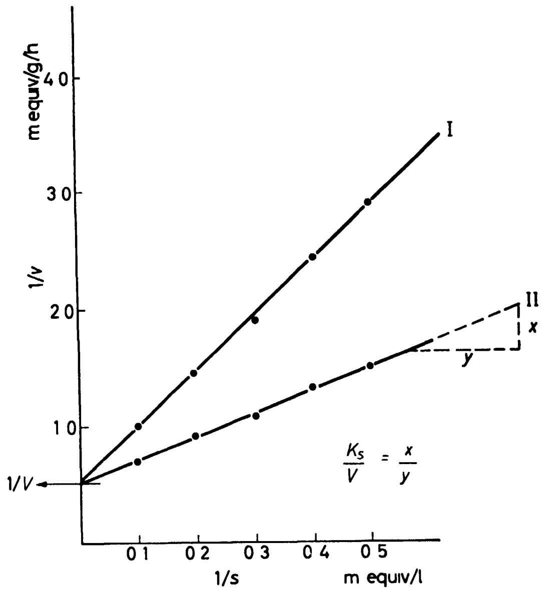


Figure 1.4. Plot of the reciprocal of ion concentration ($1/s$) against the reciprocal of the rate of uptake ($1/v$) for uptake of rubidium (I) from rubidium bromide and (II) from rubidium bromide in presence of potassium chloride (25 m equiv/l.). Conclusion can be drawn that Rb^+ and K^+ compete for the same carrier (= enzyme)

decomposition of the lecithin. Regeneration of the carrier following resynthesis of lecithin would involve the utilization of respiratory energy. The second hypothesis was suggested by the properties of the contractile protein (myosin) of muscle. The molecules of this fibre-like protein can exist in both a contracted and an extended form. The extended form is energy-rich and may spontaneously contract; the unfolding of the contracted form consumes energy from the simultaneous enzymic decomposition of an energy-rich phosphate compound (adenosine triphosphate) formed during respiration. In 1952 Goldacre[19] reported rhythmic movements of root hair vacuoles and suggested that they arose from ordered contraction and unfolding of protein molecules orientated in the vacuole membranes. Such a protein could, when its molecules are in the unfolded form, bind ions by free valencies exposed at the membrane surface. Contraction would draw these ions through the membrane and the act of contraction could lead to liberation of the ions as the free valencies of the protein became satisfied amongst themselves in the folded molecules. Energy, directly derived from respiration, could then unfold the protein and reset the trap (*Figure 1.5*). An attractive feature of this difficult-to-test hypothesis is that it clearly defines two states of the carrier, interconverted by use of a known energy source (adenosine triphosphate) and explains transport across the thickness of the membrane by making this a forceful displacement of the ions.

So far this discussion of salt absorption by roots has been confined to considering the mechanism of accumulation by the root cells. However, the root cells not only individually absorb salts but together form an organ which supplies the salt requirements of the shoot. For this there must be transport of ions across the root diameter and into the conducting cells. Steward and his associates[20], working with barley roots, found that the most active ion uptake took place at the apical growing points of the roots and that it was from this region that salt was most rapidly translocated to the

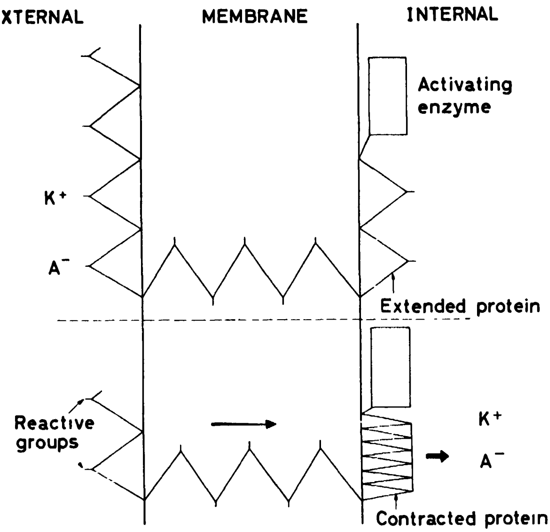


Figure 1.5. Diagrammatic representation of the Goldacre concept of the functioning of a contractile protein as a carrier of ions[19]. Energy would be fed into the system to extend the protein fibre and re-expose reactive groups external to the membrane.

shoot. In other roots, however, salt uptake may also take place rapidly both in and beyond the root hair zone. The movement of ions across the root cortex probably does not involve entry into the cell vacuoles but is mediated via the cell walls and protoplasm. The existence of protoplasmic strands connecting the protoplasts of adjacent cells means that there is a protoplasmic continuum stretching from the outermost to the innermost layer of lining cells. Movement of ions in this continuum could be by diffusion of free ions or ion complexes aided by protoplasmic streaming. At the endodermis, the Casparian strip may ensure that the protoplasmic continuum is the only pathway into the central tissues. By such a pathway salts could reach the living cells adjacent to the dead conducting cells of the xylem. The most satisfactory explanation of the further movement of ions towards the shoot that can, at present, be advanced, is that at this point there is an active secretion of salts into the transpiration stream. Comparison of the salt compositions of the root cells and of the xylem fluid points to this being

a selective secretion. At present no satisfactory explanation can be advanced as to what controls this secretion, both as regards its magnitude or ionic balance. To say that the growth activity and associated salt requirements of the shoot cells strongly influence the salt supply which they receive from the root is to describe rather than explain what happens. The nature of the stimulus which passes from shoot to root is at present unknown.

Highly calcareous soils support a flora which contrasts markedly in species composition with that of acidic base-deficient soils. At least some 'chalk' or calcicole species seem to be chemically determined; they flourish in a calcareous soil not because of its physical nature but because of a high base content associated with a high ratio of calcium and magnesium to the monovalent cations. Similarly, many typical 'acid' or calcifuge species appear intolerant of lime but able to grow well and absorb their total cation requirement (including calcium) from base-deficient soils. These observations suggest that the roots of calcicole and calcifuge plants differ in their affinities for the divalent cations, calcium and magnesium. It has also appeared from many experiments involving growth of different species in nutrient solutions, that each species has a characteristic acid-alkali range (pH range) within which it can grow, and a narrower optimum pH range at which it grows luxuriantly. However, recent work by Olsen[21], of the Carlsberg Laboratory, Copenhagen, suggests that these pH ranges are determined by iron solubility and not by the direct effects of hydrogen and hydroxyl ions. Plants characteristic of acid soils developed iron deficiency under neutral conditions; basic soil plants showed symptoms of iron toxicity (too great an uptake of iron) under acid conditions. Chelating agents which form stable soluble complexes with iron decrease the concentration of free ferric ions under acid conditions and retain iron in solution under neutral conditions. Because of this the addition of chelating agents to nutrient solutions enables both acidic and basic soil plants to grow actively over the same wide range of pH. From these interesting observations it may be suggested that the roots of acidic soil plants have a low, and those of basic soil plants a high affinity for iron. The differing affinities of plant roots for different ions, and the differing affinities as between species for the same ion are presumably determined by the chemical nature of the operative ion carriers.

Absorption of Organic Substances

Soil organic matter is clearly important in relation to soil texture, the water and salt retaining power of soils and for the growth of soil micro-organisms. The soil micro-organisms effect the decomposition of soil organic matter and the release of its mineral elements as soluble ions. The role of humates and other organic substances in increasing the availability to plants of the essential micro-nutrient elements (copper, zinc, manganese and molybdenum) has been stressed by a number of authors. Much more controversial is the question of the importance to vigorous plant growth of their absorption of soluble soil organic substances.

The ability of green flowering plants to grow vigorously and to complete their life-cycles over successive generations when supplied only with inorganic nutrients is supported by numerous experiments. In a number of such

experiments, precautions have been taken to reduce organic matter contamination to a very low level. This has led many workers to consider that growth stimulations resulting from additions of organic substances to nutrient solutions are due to influences on salt availability and not to any special nutrient value of the organic compounds. Clearly, however, such experiments do raise the following questions: (*a*) Is it not to be expected that whenever the environmental conditions are not ideal then one or more partial deficiencies will occur of organic substances synthesized by the plant and essential for its growth? (*b*) Do such compounds occur in soils in significant amounts? (*c*) If they are present, can they be absorbed by plant roots?

The ability of green and albino flowering plants to grow in darkness when supplied with sugar via their roots is the extreme case of an answer to the first of the questions set out above. However, although almost all classes of natural organic compounds, including sugars, have been detected in soils they normally occur in the soil solution at concentrations far too low to act as major sources of carbon and energy for higher plants. Further, although amino acids in mixed solutions are not inhibitory to plant growth, can be absorbed and can, in the absence of nitrates or ammonium salts, function as effective sole sources of nitrogen, it seems almost certain that in fertile soils almost all the soluble nitrogen is in the form of inorganic ions. The liberation of amino acids by leguminous plants has been suggested as the cause of the beneficial effect of legumes in mixed cropping. However, again the effect is probably indirect, the liberated amino acids being first acted upon by soil micro-organisms. Therefore, it seems most likely that if soil organic matter supplies organic nutrients of importance for the growth of green flowering plants, then the substances involved must be effective in small amounts. Such substances might be particular amino acids or nitrogenous bases, vitamins, plant hormones and other organic substances known to be essential for or markedly stimulatory to the growth of certain micro-organisms including some algae. Schreiner and his co-workers at the Bureau of Soils of the United States Department of Agriculture, have demonstrated the presence in fertile soils of certain amino acids, purines and other simple organic nitrogen compounds. Appreciable quantities of vitamin B_1 have been detected in a number of organic manures and such manures have been claimed to enhance the vitamin content of crop plants. Bacteria producing substances with the physiological activity of the plant growth hormone, auxin (β-indolylacetic acid, IAA) have been isolated from soil extracts by various workers. Stewart and Anderson[22] obtained evidence of a relationship between the auxin content of soils and their fertility. In view of these and many other similar investigations, it seems clear that this whole aspect of the soil–root relationship should be further investigated.

In order to demonstrate unambiguously that organic substances can be absorbed by roots it is necessary to exclude contamination by micro-organisms which could cause their chemical modification, destruction or removal from solution. As early as 1909, Hutchinson and Miller[23] used sterile seedling cultures to study the ability of amino acids to supply the nitrogen requirements of plants. Techniques for the sterile growth of plants have since been further elaborated and have demonstrated that roots can

absorb a very wide range of soluble organic substances. Sterile root cultures first successfully established by White[24] at the Rockefeller Institute for Medical Research and by Robbins at the New York Botanic Garden, have also extended our knowledge of this aspect of the absorbing capacity of the root system. Quite irrespective of the importance or otherwise of organic soil nutrients for plant growth, such studies could contribute to our understanding of the mechanism of transport within the plant of organic substances and of the secretory activity of plant glands like nectaries.

Certain substances are known which specifically interfere with the conservation in utilizable form of the energy liberated in respiration. Such substances, of which dinitrophenol is an example, are termed 'uncoupling' agents because they uncouple respiration from the reactions which lead to the synthesis of those phosphorus compounds (such as adenosine triphosphate) which act as sources of energy for cellular activity. Such 'uncoupling' agents not only inhibit the growth of root cultures but also the uptake of inorganic ions, amino acids and sugars by the cultures. Respiratory inhibitors similarly inhibit the accumulation of inorganic ions and organic nutrients by root cultures. Further, certain general inhibitors of enzymes (for example uranium salts) have proved very potent inhibitors of sugar uptake when applied under conditions preventing their penetration into plant cells. It can be postulated that such substances inhibit sugar uptake by inactivating 'carriers' at the cell surface.

Studies with seedling roots and root cultures[25], of the influence of concentration upon sugar uptake and of the uptake of individual sugars from mixed solutions, have shown that different sugars have different maximum rates of uptake, and have demonstrated both selective sugar absorption and competitive effects between sugars. Similar problems are posed by these studies to those discussed when considering the accumulation of inorganic ions and here again a major advance in our understanding of the accumulation process requires the chemical identification of the forms in which these solutes are absorbed into root cells.

Mycorrhizas

A more or less definite association of the root system with a fungus, the development of a mycorrhiza (fungus–root), is probably, under natural conditions, as usual as the uninfected state. Frank, to whom we owe the term 'mycorrhiza', first described the enclosing fungal sheath normally present on the roots of both flowering and conifer trees growing in established woodlands. The existence of this well-developed enclosing sheath and the fact that the fungal hyphae penetrate between rather than into the root cells has led to the classification of such mycorrhizas as ectotrophic. This is in contrast to the more numerous, more variable and, at least in some cases, less well authenticated mycorrhizas in which the sheath is absent or less well developed and in which there is always evidence of intracellular hyphae; such mycorrhizas are termed endotrophic. The use of the phrase 'less well authenticated' emphasizes that for many endotrophic mycorrhizas the nature of the physiological relationship between the fungus and the root is obscure. The pioneer work on mycorrhizas stressed that a symbiotic relationship was

involved, both partners benefiting from the association, and this is clearly the case in ectotrophic and in some endotrophic mycorrhizas.

It would clearly go beyond the scope of the review to give a general account of mycorrhizas. However, it is equally impossible to present a balanced assessment of the absorbing functions of the root without considering the experimentally verified knowledge we now have of the physiological significance of mycorrhizas[26]. It is, therefore, this aspect of the root–fungus interaction which is now briefly reviewed.

The fungi of the ectotrophic mycorrhizas of woodland trees are, in many cases, known to be Basidiomycetes (gill-bearing fungi). They differ from the other forest litter-inhabiting members of this group in being unable or in having only a very limited capacity to decompose the cell wall materials (lignin and cellulose) of the decaying plant organic matter. These mycorrhizal fungi when isolated and grown in culture are all readily able to utilize simple sugars such as glucose but only some can utilize more complex sugars or starch. They do not readily utilize nitrate but can assimilate the nitrogen of ammonia, amino acids, urea and other simple organic nitrogen compounds. Often particular amino acids, for instance glutamic acid, very actively stimulate their growth. Most of these fungi have vitamin requirements, notably for vitamin B_1 and other members of the B group (*I–V*) (p. 18) and their growth is further stimulated by unknown growth factors generally present in root extracts and exudates. When the infected higher plant is allowed to carry on photosynthesis in the presence of radioactive carbon dioxide, the organic constituents of the fungal hyphae become radioactive suggesting that the higher plant supplies at least part of the requirements of the fungus for organic nutrients.

Rootlets with ectotrophic mycorrhiza possess four 'tissue' systems: (*a*) an external system of hyphae in the soil, (*b*) the fungal sheath around the root, (*c*) hyphae running between the cells of the root cortex (this forms the Hartig net), and (*d*) the cortical and stelar cells of the root itself. The external system of hyphae has been shown by Melin and his associates in the University of Uppsala to absorb phosphate, calcium and nitrogen compounds, and these have subsequently been traced as they are passed on to the roots and ultimately the shoots of the host seedlings. The active uptake of ions by the external hyphae depends upon oxygen availability and a suitable temperature, and both these factors are at a favourable level in the upper horizons of woodland soils where the mycorrhizas are most evident. Studies of phosphate uptake by excised mycorrhizal roots have shown the ability of the mycorrhizal sheath to accumulate this ion rapidly from dilute solutions. However, only 10 per cent or less of the phosphate is passed on to the root cells during the uptake period. The fungal sheath is a very active accumulator of phosphate, but it is also very retentive of the absorbed ions. If mycorrhizas which have accumulated phosphate are then transferred to phosphate-free solution or to solutions of very low ionic strength, a slow but continuous transfer of phosphate from the sheath to the root cells occurs. These two observations may be the key to understanding the importance of mycorrhizal infection for the active growth of forest trees in soils of low mineral status. The release of soluble material from the decaying litter of the woodland floor is not a steady process; there are periodic flushes of

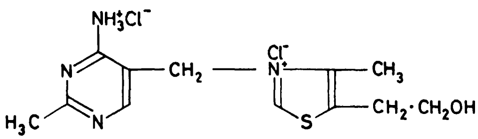
nutrients from newly fallen litter. Infected roots may selectively absorb and accumulate essential nutrient ions released in such flushes more efficiently than do uninfected roots, and then we can visualize the enriched fungal sheath acting as a salt reservoir which gradually transfers ions to the tree roots at times when the ionic content of the soil solution is very low. It has also been suggested that mycorrhizal roots, because of their drought and frost resistance, have an enhanced ability to persist in the surface layers of the soil under unfavourable climatic conditions.

The endotrophic mycorrhizas of plants of the heath family (Ericaceae) clearly resemble the ectotrophic mycorrhizas of forest trees in the development of external mycelium and in being most clearly beneficial and most evident in soils of low ionic status. Although it cannot be ruled out that the fungi pass on organic substances derived from soil humus, the balance of evidence is that ericaceous mycorrhizas promote plant growth primarily by increasing the uptake of inorganic ions from poor heath soils. There is no good evidence that the fungi are nitrogen-fixing organisms. The mycorrhiza of the chlorophyll-free Yellow Bird's-nest (*Monotropa hypopithys*) also in many ways, resembles morphologically the mycorrhizas of forest trees except for the frequency of hyphae penetrating the root cells. In this case, however, the mycorrhiza clearly absorbs and transmits to the flowering plant not only inorganic but also the organic nutrients which in the absence of chlorophyll it cannot synthesize for itself. For these and many other endotrophic mycorrhizas we have, however, very incomplete knowledge concerning the identity of the fungi, or of their growth requirements, absorbing capacities and ability to form humus-degrading enzymes.

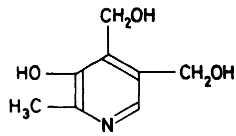
The orchids have always exercised a peculiar fascination for botanists and our extensive knowledge of the development of their endotrophic mycorrhizas and of the fungi concerned is based on the brilliant classical researches of Noel Bernard and Hans Burgeff. Orchids either pass through a stage when they lack chlorophyll and therefore require to receive organic nutrients or this is so throughout their growth (*i.e.* they remain saprophytic). Mycorrhizal infection essential for the saprophytic growth starts at the earliest stage of the germination of the minute orchid embryo. As the plant grows the tuberous roots and the aerial green roots, if present, remain uninfected. By contrast, the absorbing roots are mycorrhizal and here the fungus continually invades the new cells as the intracellular hyphae are digested in the older cells. The extent of infection as might be expected remains high in saprophytic orchids; in those which become photosynthetic there is a recession in the extent of the fungus infection as the chlorophyll-containing tissue develops and the plant may become, eventually, virtually fungus-free.

The fungi concerned in orchid mycorrhizas have in nearly all cases of certain isolation proved to be 'imperfect' Basidiomycetes. The isolates have proved to be easily grown in culture. They are able to utilize a wide range of carbon compounds including lignin, cellulose and other polysaccharides which they degrade by liberating from their mycelia the appropriate enzymes. They do, however, resemble the fungi of ectotrophic mycorrhizas in their poor ability to utilize nitrate, in their active accumulation of ammonia and simple organic nitrogen compounds and in their requirements for vitamins of the B complex.

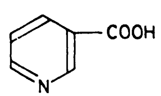
It is possible that the orchid plant absorbs sugars and growth factors directly from the humus layer of the soil as these are formed there by the activity of fungal enzymes. However, direct transfer across the fungal walls into the orchid cells seems more likely and it is certain that the digestion of fungal hyphae within the orchid cells, although perhaps primarily an



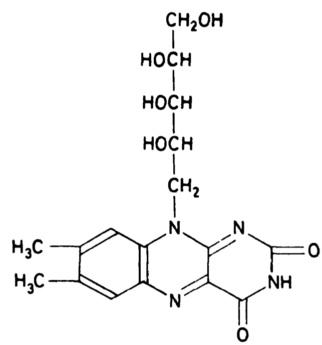
(I) Thiamine hydrochloride
(vitamin B_1)



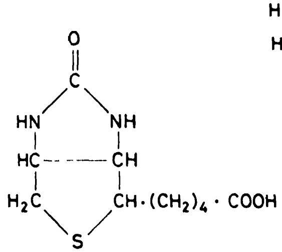
(II) Pyridoxine
(vitamin B_6)



(III) Niacin
(nicotinic acid)



(IV) Riboflavin
(vitamin B_2)



(V) Biotin

expression of the control of infection, leads inevitably to a release of nutrients directly into the orchid cells. The role of the fungal partner in the nutrition of the orchid plant probably varies as between species. The classical studies of Knudson, of Cornell University, on the essentiality of sugar for the successful germination of uninfected orchid seeds showed that for some few species this was the only essential organic nutrient. Many others had additional essential nutrient requirements which could be met more or less effectively by plant decoctions, extracts of the mycorrhizal fungus, yeast extracts and so on. The activity of such extracts seemed to be due to their vitamin content, particularly their contents of vitamins B_1 and B_6 (*I* and *II*)

and niacin (*III*). No corresponding studies have been made of the importance of orchid mycorrhizas in the absorption anc transport to the orchid plant of inorganic ions.

In addition to the morphologically well-defined mycorrhizas so far considered there are many less obvious root–fungus relationships of a mycorrhizal type, and amongst these are the widespread associations of the roots of woody and herbaceous plants with fungi whose hyphae typically lack cross walls or septa. These aseptate fungi belong apparently to the two genera, *Endogone* and *Pythium*, both of which are members of the group called Phycomycetes. Mycorrhizas involving these fungi are often referred to as vesicular-arbuscular because the main hyphae often show characteristic swellings or vesicles and the hyphae which penetrate the root cells, form complex branch systems or 'arbuscules' (bush-like systems). There is never any sort of sheath of fungus on the root surface, but recent work has demonstrated the presence of a loose external weft of hyphae which extends into the soil and is connected with the internal mycelium via the piliferous layer of the root. Something is known regarding the growth requirements of the fungi, and digestion of those hyphae which penetrate the root cells has been observed. Experimental work on the role of these mycorrhizas in plant nutrition is, however, lacking. This is particularly to be regretted because many plants of great economic importance, crop plants and timber trees, possess these phycomycetous mycorrhizas.

THE EXCRETION OF SUBSTANCES FROM ROOTS

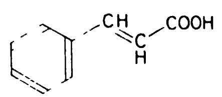
The soil around plant roots is always characterized by a microflora which is qualitatively different from and more abundant than that in the general body of the soil. This region of the soil under the influence of plant roots, with its high density of bacteria and enhanced population of free-living fungi is known as the 'rhizosphere'[27]. Under soil conditions, root hairs are ephemeral structures and the root cap constantly sloughs off dead cells. The development of cork on the older parts of the root system leads to the death of the outer tissues of the root. These processes inevitably yield root cell constituents to the soil. The demonstration that young seedlings have a rhizosphere effect does, however, point also to the excretion of organic substances from living root cells and a number of workers have considered such excretions to be the primary reason for the establishment and maintenance of the rhizosphere.

Bacteria for which amino acids are essential nutrients have an enhanced prominence in the rhizosphere and this is often particularly so in the rich rhizosphere of legume roots. This is thought to be due to the excretion of amino acids by roots, and in all some 17 different amino acids and the amides, glutamine and asparagine, have been detected as exudates from seedling roots and root cultures. The nodulated roots of legumes have also been reported to liberate, at least under certain environmental conditions, quite exceptional amounts of amino acids, particularly of glutamic and aspartic acids.

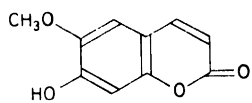
Sugars, particularly glucose and fructose, are liberated by roots but apparently in amounts too small to be of physiological significance in the

soil. Seedlings and root cultures of certain species secrete the B vitamins, particularly riboflavin and biotin (*IV* and *V*), but such vitamin liberation does not seem to be important in the rhizosphere where, if anything, there is an enhancement in the proportion of vitamin-synthesizing bacteria. We have, however, already presented data suggesting that vitamin supply by the root to the fungal partner is important in the mycorrhizal relationship. Nucleotides (adenine, guanine, uridine and cytidine) have also been identified in root exudates, but knowledge of the growth requirements of soil bacteria does not yet permit of a final assessment of their role in the rhizosphere effect.

There is a considerable body of experimental evidence that root exudates are important in nature not only through their influence on the soil microflora but also in the interaction between flowering plants. Borner has recently demonstrated both an inhibition of the growth of mustard (*Sinapis arvensis*) by oats (*Avena sativa*) and a self-intolerance of flax, under conditions eliminating competition for space, light and mineral salts. Many similar, if less critical experiments have been previously reported. However, such experiments cannot be satisfactorily interpreted until the operative agents have been chemically identified. In those few instances where toxic root excretions have been chemically identified there is considerable doubt as to their significance in natural competition. For instance, the roots of mature guayule plants (*Parthenium argentatum*) were shown by Bonner[28], working at the California Institute of Technology, to secrete *trans*-cinnamic acid (*VI*) and this inhibited the growth of seedling plants of this species at a concentration of 1 part per million. However, under natural soil conditions it seems that the *trans*-cinnamic acid is decomposed by soil bacteria as rapidly as it is liberated and that no effective soil toxicity to seedlings is ever established. Seedling roots of oats secrete fluorescent substances including scopoletin (*VII*) and its glycoside, and these are potent inhibitors of the growth of roots of oats and other grasses and are also germination inhibitors. There is, however, no evidence that the natural secretion of these compounds ever establishes an effective inhibitory concentration in the soil.



(*VI*) *trans*-Cinnamic acid



(*VII*) Scopoletin

One even more controversial aspect of root excretion should be mentioned. There are a number of reports, mainly by Russian scientists, that enzymes are secreted into the soil not only by mycorrhizas but also by 'uninfected' roots. However, as previously described, many plant roots form associations with fungal hyphae which do not reach the status of morphologically obvious mycorrhizas, and we still await the demonstration of a liberation of enzymes from sterile root cultures. There is evidence, however, for excretion of enzymes from higher plant cells in insectivorous plants and Nickell has described the secretion of the enzyme amylase by a sterile culture of sorrel tumour tissue. It has also been suggested by Rogers and his associates[29]

that enzymes excreted by the root play a role in the phosphorus nutrition of plants by breaking down the natural organic phosphorus compounds of soils.

Our knowledge of the excretion of organic substances by root cells, although in certain respects quite extensive, does not extend to the physiological level. Only future quantitative studies under controlled conditions can reveal the nature of the controlling factors and open the way to an understanding of the operative physiological mechanisms.

THE SHOOT-ROOT RELATIONSHIP

Green plants, by virtue of their ability to synthesize organic compounds from carbon dioxide, water and inorganic ions, can complete their life-cycles in an inorganic environment; they are autotrophic. The photosynthesis of sugars proceeds only in the green chlorophyll-containing tissues. In land plants the absorption of mineral nutrients is confined to the chlorphyll-free tissues of the root. The separate organs of the higher plant are nutritionally interdependent. The many specialized tissues may be expected to each have their special nutrient requirements. Some tissues are certainly heterotrophic, others probably so (including the photosynthetic tissues); that is, they are dependent upon other tissues for one or more essential organic nutrients.

Such nutritional interrelationships in complex organisms will exist between the individual cells of tissues, between the different tissues and between the separate organs. It is, therefore, relevant to our study of the physiology of roots to consider their nutritional relationships with shoot systems. In a limited and qualitative way this can now be attempted as a result of studies on the growth requirements of isolated roots[30]. Such studies are, as yet, limited in number and scope, and furthermore, conclusions drawn from them are based on the assumption that the nutritional requirements of isolated roots in culture reflect their requirements in the intact plant. In support of this assumption we may point to the normality of morphology and minute structure maintained by cultured roots. Furthermore, reservations regarding the relevance of such studies to the interpretation of the normal shoot–root relationship are presumably susceptible in some measure to future test by whole plant studies involving the appropriate use of isotopic tracers.

As would be expected, excised roots require for growth in culture, a supply of a utilizable sugar. The pioneers of excised root culture envisaged this requirement and incorporated glucose into their nutrient solutions. In these early experiments, undertaken in 1922–3, it was shown that excised seedling root tips of maize, wheat and several other species showed hardly any growth in a sugar-free culture solution, but that the addition of glucose promoted a high initial rate of growth. However, even in the presence of glucose, this growth was not maintained and the cessation of growth was speeded up when attempts were made to initiate secondary cultures by cutting off (excising) and transferring to new solution the original radical tip or any lateral tips initiated during culture. However, when in 1932 White substituted sucrose (cane sugar) for glucose in the nutrient solution, he was able, for the first time, to establish continuous cultures of excised tomato roots. The roots could be subcultured indefinitely and without

diminution of growth rate by excising root tips (about 1/3 in. long) from the lateral roots developed in culture and transferring these to new culture solution (*Figure 1.6*). The use of sucrose as the primary supply of carbon and energy has subsequently been shown to promote the continuous growth of root cultures of a number of dicotyledonous species. With such species, glucose and fructose initially support rates of growth lower than that with sucrose and do not permit the roots to be repeatedly subcultured. The

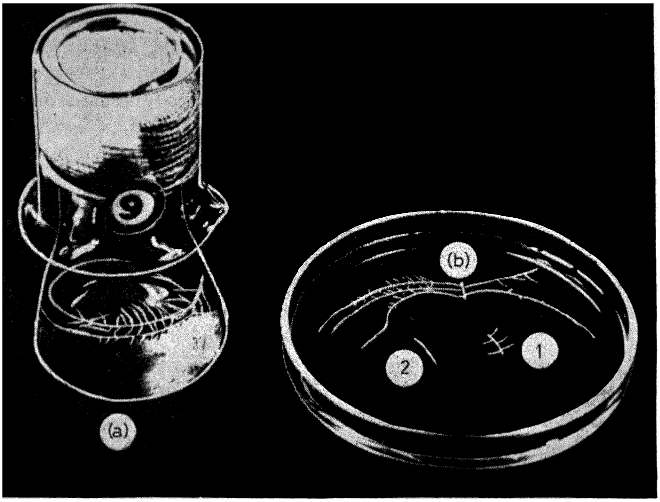


Figure 1.6. Technique used for the maintenance of clones of excised tomato roots
(*a*) 100-ml. culture flask containing a 6-day-old tip culture. (*b*) A sector culture: (*1*) The inoculum used to start off a sector culture. This is a portion of the main axis of a tip culture and bears 5 lateral roots, each 3 to 5 mm long. (*2*) A root tip (10 mm long) used to start off a tip culture such as that shown in (*a*)

hydrolysis of sucrose splits it into a mixture ('invert sugar') of its two constituent sugars, glucose and fructose. This 'invert sugar' is not equivalent to sucrose as a source of carbon and energy for the growth of root cultures. The reason why sucrose is a unique sugar for the growth of root cultures has not been satisfactorily explained. It is, however, perhaps not a surprising finding for sucrose is the first free sugar formed in photosynthesis and it is probably almost entirely as sucrose that carbohydrate is transported from the shoot to the root via the phloem tissue.

With excised tomato root cultures the linear growth and increase in dry weight of root material is about 10 times as great with sucrose as with glucose, and in an attempt to explain this, comparative studies of the utilization of sucrose and glucose have been undertaken[25]. Glucose does not exert any inhibitory effect in the presence of an adequate supply of sucrose. Nor is it true that the roots can meet the major part of their carbon requirement from glucose but require catalytic amounts of sucrose. Using roots

grown with sucrose and subsequently depleted of their internal sugar reserves by a period of starvation in sugar-free medium, it has been shown that sucrose is absorbed at a significantly higher rate than glucose. The uptake of glucose is, however, sufficient for it to support as high a rate of respiration as does sucrose. Furthermore, the absorption of radioactive glucose can be shown to lead to the formation in the root cells of radioactive sucrose. The big difference seems to be that sucrose-feeding enables a significantly higher level of sucrose to be established in the cells than does glucose-feeding. This higher level of internal sucrose may be critical either for the rate of some aspect of cell synthesis or for the supply of sugar to the growing cells at the root apex. The latter possibility is emphasized by work indicating that the mature root cells accumulate sugar from the nutrient solution much more actively than do the growing cells of the root tip. Studies of the incorporation of radioactive carbon into other cell constituents from sucrose and glucose and studies of the level of radioactive carbon established in the apical growing cells following sucrose and glucose-feeding are now in progress, and should lead to an important advance in our understanding of the sucrose requirement of these dicotyledonous root cultures.

Although cereal roots were used in the first attempts to grow excised roots, they have proved peculiarly resistant to continuous culture. The only exception to this, in fact the only monocotyledonous root which has been obtained in continuous culture, is that of Petkus II winter rye. This problem of the resistance to culture of cereal roots will be referred to later. The point to be made here is that Petkus II rye roots grow slightly better in glucose than sucrose and that other cereal roots do, in general, make somewhat more total growth and show higher initial growth rates with glucose than with sucrose. If the work with tomato roots discussed above is relevant to the problem of sugar utilization by these monocotyledonous roots it may be suggested that they, in contrast to the dicotyledonous roots so far studied, absorb glucose more readily than sucrose. In consequence, a higher level of internal sucrose may occur when the root is grown in a culture solution containing not sucrose itself but glucose. This hypothesis, however, still awaits experimental confirmation.

Whereas sucrose, glucose and fructose have variable root growth-promoting activity, all other natural sugars which have been tested are almost or quite inactive as carbon sources for excised root growth. In most cases this cannot be explained on the grounds that they are not absorbed by root cells. The simple sugars, mannose and galactose, are particularly inhibitory to root growth and in both cases the growth inhibition can be specifically reversed by incorporating simultaneously into the culture solution sufficient glucose. Galactose, although strongly inhibitory to growth, can be respired and does not retard the active respiration occurring in the presence of sucrose. It seems to interfere with the normal growth of the cell walls of the root cells. Mannose is, by contrast, a strong inhibitor of respiration and only by supplying sufficient external glucose can this inhibition be reversed. Our inability to explain in chemical terms the mechanisms of these growth and respiratory inhibitions emphasizes that we have much yet to learn concerning the interrelationships between the sugars in plant cells.

The culture solution in which White first established excised tomato roots in continuous culture contained sucrose, mineral salts and 0·01 per cent of pasteurized brewer's yeast. The yeast is rich in vitamins of the B group and White showed that its effect on root growth could be fully reproduced by a mixture of thiamine (*I*), pyridoxine (*II*) and niacin (*III*). The roots appeared to have an absolute requirement for thiamine, and in its presence a further marked improvement in growth followed the addition of the other two B vitamins. Studies with the roots of a number of other species have also revealed requirements for these B vitamins.

In most cases the addition of thiamine not only markedly stimulates growth but is essential for continuous culture. However, in the case of flax and white clover roots it has been shown that they synthesize sub-optimal[31] amounts of thiamine and can maintain a reduced rate of growth in its absence. This has been taken to indicate that the absolute requirement for an external supply of thiamine shown by the excised roots of most species arises from a complete inability of the root cells to synthesize this vitamin. However, the possibility that the critical deficiency arises despite a very low rate of synthesis has not been eliminated. With pyridoxine and niacin the position is that according to the species of root chosen the action of each vitamin may vary from being slightly stimulatory to growth, up to it being quite essential for continued growth of the roots in culture. Clearly, in the excised roots of some species it would seem that these vitamins are synthesized and that the discrepancy between the amount synthesized and the optimum requirement varies widely. Excised roots from selected species or varieties should, therefore, be particularly valuable material not only for studying the influence of environmental and nutritional factors in the synthesis of the B vitamins but for tracing the chemical pathways involved in their synthesis.

The possibility cannot be completely excluded that excised roots have an impaired ability as compared with the roots of intact plants for the synthesis of these vitamins. There is, however, evidence from whole plant studies that thiamine and pyridoxine synthesized in the shoot system are translocated to the root system via the phloem and that this supply from the shoot is important for root growth. Further, although excised roots show variable degrees of heterotrophy for thiamine, pyridoxine and niacin, they readily synthesize certain other members of the B vitamin complex. Biotin (*V*) has been shown to be synthesized by roots of flax, white clover, alfalfa and tomato; riboflavin (*IV*) by roots of white clover, alfalfa, tomato, *Datura* sp. and sunflower and *p*-aminobenzoic acid by tomato roots. Further, although direct assays have not been carried out, other B vitamins such as pantothenic acid, inositol and folic acid have proved to be without stimulatory effects on excised root growth and are, therefore, probably synthesized in adequate amount.

Isolated root systems are, in general, able to synthesize all their essential organic nitrogen compounds including proteins from nitrate. An active nitrate-reducing system of enzymes has been demonstrated in excised tomato roots[32] and there is evidence that in a number of plants the main site of nitrate reduction is the root system[33]. Additions of amino acids either singly or in combination have, in the presence of nitrate, proved to be, according to concentration, either without effect or inhibitory to the growth

of the excised roots of most of the limited number of species so far tested. However, addition of arginine or any of a number of amino acids related to arginine has been found to stimulate significantly the growth of excised roots of groundsel. The amino acids, glycine and cystine, are also stimulatory to excised root cultures derived from certain strains of tomato. Tryptophan (*X*) (p. 38) is rather exceptional in that an external supply of this amino acid seems to be very markedly stimulatory to growth or even essential for the continued growth of certain cereal roots. On present evidence, however, this activity of tryptophan seems to arise from it being a compound readily transformed into a growth hormone or auxin rather than from the inability of the responsive roots to synthesize the tryptophan required as a unit of structure in their proteins.

With a less satisfactory nitrogen source than nitrate, stimulatory effects of externally applied amino acids are more easily demonstrated. The growth of root cultures supplied with inorganic nitrogen as an ammonium salt can be significantly enhanced by amino acid additions, particularly by additions of simple mixtures of the basic amino acids. A number of simple organic nitrogen compounds other than amino acids have also been tested for their growth effects on root cultures. All such compounds have, in presence of nitrate, proved inactive or inhibitory to root growth. It may be, of course, that as the growth requirements of the excised roots of more species are described there will emerge more and better evidence of the heterotrophy of roots in relation to amino acids or other organic nitrogen compounds. However, the present limited data do rather suggest that roots receiving a utilizable sugar and a satisfactory source of inorganic nitrogen are active centres for the synthesis of the large number of organic nitrogen compounds essential to living cells. If this is so, then the few instances of amino acid stimulation which have been recorded with root cultures would be subject to the general interpretation that they simply reflected the operation of conditions of culture unfavourable for amino acid synthesis in the root cells.

Studies with land plants have shown that the essential chemical elements required for growth, with the exception of carbon are normally obtained as water and as inorganic ions entering through the root system. This raises the question of whether the elements required for root growth are identical with those required for the whole plant. Studies undertaken with excised roots of tomato go far towards answering this question. Pioneer work by White demonstrated the essentiality of calcium, magnesium, potassium, iron and inorganic nitrogen. White also demonstrated a marked reduction of growth following omission of sulphate and phosphate and the essentiality of sulphate and phosphate has been demonstrated in our own laboratory. From our knowledge of the chemistry of cell constituents such findings could be confidently predicted. However, plants also require a number of micronutrient elements which are effective in very small amounts and whose function in cell physiology seems to be as constituents of enzymes. Certain enzymic reactions proceed only in shoot cells (for instance, certain photosynthetic reactions) and the products of such reactions will, therefore, be organic nutrient requirements of the root. If this is so, certain micronutrient elements essential for whole plant growth may not be required by

excised roots growing in a medium supplying those organic substances normally received by translocation from the shoot. When, in 1947, Glasstone obtained evidence that iron and copper were the only essential micronutrient elements for the growth of excised tomato roots she followed this interpretation, and concluded that the apparently simpler requirements of the root as compared with the whole tomato plant were 'not surprising'. However, subsequent work in our laboratory, particularly by Hannay, has demonstrated the essentiality of manganese and molybdenum for the growth of excised tomato roots, and more recently, the work of Neales with excised roots of flax has made it very probable that boron is also essential. The only well-established plant micro-nutrient whose essentiality for root growth is still in serious doubt is zinc, and even here, the most recent work suggests that the demonstration of essentiality waits upon an improvement of the techniques for removing zinc contamination from the other constituents of root culture solutions. The balance of evidence, therefore, strongly suggests that all or most of the enzymes which are activated by the micro-nutrient elements are essential constituents of root cells.

Growth factors and hormones are today preoccupations of plant physiologists. There is, however, much confusion in the use of these designations. Growth factors can be defined as organic substances which, in minute quantities, significantly promote growth. Many such growth factors are essential natural constituents of cells. If the synthesis of such natural growth factors is impaired an appropriate external supply will usually promote growth; if the ability to synthesize one or more such factors is lost by a change in the hereditary material (by mutation) then an external supply is essential for the life of the organism. The vitamins, previously referred to, are natural growth factors. The term, hormone, comes originally from animal physiology and is used for natural organic substances active in minute amount and exerting their characteristic physiological effects at sites remote from those at which they are synthesized. Where the physiological effect is particularly upon the growth of the organism it is a growth hormone —a natural growth factor in which synthesis and growth control are spatially separated. This designation 'growth hormone' came into common use in plant physiology following the discovery of the auxins[34]. It has now been extended by many authors to include other natural growth factors which control cell division and cell enlargement (such as the gibberellins) although in all cases, including that of the auxins, we now know that there is no sharp spatial separation between the sites of synthesis and physiological activity.

We understand the nature of the essential physiological role of some growth factors. The vitamins are essential parts of certain enzymes. Others, like the amino acids, are essential building bricks for larger and equally essential cell constituents. The mechanism of action of the animal hormones has proved a uniquely baffling problem. The mechanism of action of those growth factors usually referred to, by plant physiologists, as growth hormones, is equally obscure.

In a later section of this chapter (p. 35) reference will be made to the little understood role of growth hormones (auxins and gibberellins) in the root–shoot relationship. However, at this point some reference must be made to a number of chemically unidentified plant growth factors. Discovery

of the existence of such factors followed from the incorporation of complex natural extracts or fluids (*e.g.* yeast extracts, coconut milk and other liquid endosperms, extracts of plant tissues) into culture solutions designed to maintain the growth of isolated plant tissues and organs. In a number of important instances of this kind it has not been possible to account for the growth-promoting activity of the supplement in terms of its known chemical constituents. The most dramatic growth-promoting effects of such complex supplements have been recorded in studies of the growth in culture of callus fragments derived from the new tissue which arises in response to the wounding of many plant organs. It may, therefore, be that the unidentified growth factors concerned will prove to be of general importance in the physiological relationships between individual plant tissues. However, the excised roots of a number of species have failed to grow in media of known composition but have proved amenable to culture in presence of supplements such as yeast extracts or protein hydrolysates. Further, in a few cases it has been reported that the activity of the complex supplement could not be reproduced by a mixture of its known constituents. It should also be recognized that of the excised roots of the species which have, from time to time, been tested for their culturability, more have proved resistant than amenable to continuous growth. Further studies with the excised roots of these resistant species may well expand the present list of growth factors for which roots can be heterotrophic, and hence presumably dependent upon the synthetic activity of the shoot system.

ROOT GROWTH

Meristems: the Division, Enlargement and Maturation of Root Cells

The growth of an organ like the root is the expression of an increase in the number of its constituent cells by cell division, enlargement of the new cells and their development (differentiation) into different kinds of tissue cells (xylem cells, phloem cells, cortical cells and so on). Further, these processes are localized in growing centres or meristems. In the root system, the primary meristems are at the apical tips of the roots and, in addition, there are the secondary meristems or cambia which, in the older parts of the system, give rise to additional stelar tissues and to protective layers of cork. Not only is the over-all growth of the root system the sum of the activities of these separate meristems but their relative activity determines the morphology of the system. Thus, if the apical meristem of the seedling radicle retains a high level of activity a tap root system develops; alternatively, if the level and duration of activities of this initial meristem and of subsequently formed meristems are less, then a highly branched fibrous system develops. Further, as hinted above, the activity of each meristem influences the activity of other meristems and particularly those nearest to it. Such growth correlations are involved, for instance, in apical dominance, where there is an apparently inhibitory influence of an active apical root meristem on the activity of the more recently initiated lateral meristems.

The extreme tip of the root is occupied by a cap of cells protecting and derived from a group of cells in the permanently dividing tissue or promeristem. All of the new cells formed by the growing root have their ultimate

origin in this promeristem, some arising from such cells following a limited number of further divisions. Studies[35] of fully developed root apices have resulted in much controversy regarding the number of cells constituting the promeristem. One concept which seems to apply to root tips of certain species at least, is that the promeristem is a group of dividing cells lying on the surface of a hemisphere, the proximal surface of the hemisphere consisting of a plate of cells giving rise to the stele and cortex and piliferous layer and

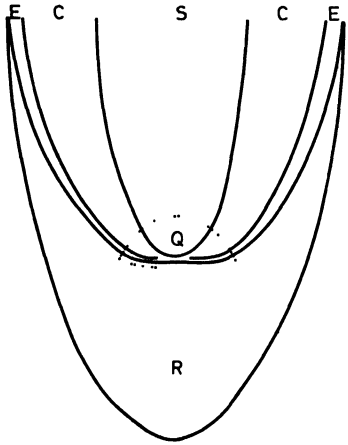


Figure 1.7. Diagrammatic median section of the root apex of maize. The sites of the initial cells of the promeristem are indicated by dots. E = piliferous layer; C = cortex; S = stele; Q = quiescent centre; R = root cap. (Drawn by Dr. L. Clowes)

sometimes also to the outer cells of the root cap. This hemisphere is filled with a group of cells (as many as 500 to 1,000 cells) which rarely divide and constitute the quiescent centre of the meristem. The distal (apical) flat surface of this hemisphere of quiescent cells is covered by a further plate of dividing cells which give rise to the central cells of the root cap (*Figure 1.7*). Apparently, both the number of dividing cells and of quiescent cells and the destiny of the newly formed cells may vary during development; the quiescent centre is absent or represented by very few cells in young lateral roots and embryonic roots.

The apical root meristem is an organized structure; there is a pattern in the arrangement of its constituent cells. This pattern is maintained by the planes along which division walls are laid down in cell division and by a balance between rates of cell division on the one hand and the rates and directions of cell expansion on the other. The orientation of the division walls is apparently in turn determined by the shape of the spindle arising during the nuclear division (mitosis) which precedes cell division. Changes in the extent of the meristematic zone or stabilization of meristem size are again determined by a balance—a balance between division rate and the

rate at which the daughter cells mature to that point where they develop directly into the tissue cells of the root.

Not only is there a pattern in the meristem but there is a patterned differentiation of the new cells into the typical tissue arrangement of the primary root. This destiny of the cells, the particular kind of tissue cells into which they develop is, apparently, determined by their position in the meristem and not by an apically directed influence emanating from the mature root tissues. If root tips are cut off, re-orientated and then replaced on the root stump, the new tissues as they appear are out of line with those of the stump. It is in the apical millimetre or so of the root that the tissue pattern is determined.

The growth of cells in the apical region of the root, to give rise to the cells characteristic of each tissue and differing in size and shape, apparently involves a gradual mutual adjustment of their cell walls 'as a common framework'. The walls of adjacent cells grow in unison. This Priestley called 'symplastic growth'. The growth of the piliferous layer of the root has, for instance, been shown to involve differential rates of growth in different parts of the wall of each cell, the apical end of each cell generally continuing its growth longer than the basal end. There is no evidence for the occurrence in the root tip of the 'gliding growth', first described by Krabbe in 1886, and in which adjacent cell walls slide past one another as they expand.

The study of organization and physiology within the promeristem is extremely difficult owing to its minute size. The repeated use of 'apparently' in the above sentences follows from the controversial nature of certain deductions based upon the microscopic study of prepared sections of root apices. The only technique by which we can at present attempt to examine differences in physiology between promeristem cells is that of radioautography. Meristems fed with radioactive substances can subsequently be sectioned and the pattern of distribution of radioisotope within the promeristem revealed by placing the section in contact with a fine-grain photographic emulsion. The film is then developed and examined microscopically superimposed upon the section. This technique, for instance, provided valuable evidence for the quiescent centre, a mass of cells in which the rate of synthesis of chromosomal and other cell proteins proceeds very slowly.

Behind the promeristem is the region where cell enlargement and differentiation are the dominant processes. The potential for growth in successive regions can be studied by the simple class-technique of Indian ink marking at millimetre intervals followed by measurement of the displacement of the marks by subsequent growth. This linear sequence of zones also represents the different growth stages through which each group of newly formed cells passes with time as it is displaced farther and farther from the root tip by the continuing addition of new cells from the promeristem. For instance, in the seedling maize root cell divisions are not observed beyond 1·5 mm from the root cap and it is in the 2nd and 3rd mm from the root cap that the most active linear extension by cell expansion takes place. By 4 mm from the root cap most of the root cells are fully expanded and already their differentiation into the root tissues is well advanced.

By studying the growth rate and by recording by microscopic observation the cell dimensions at known distances from the root apex, the time course

of cell expansion in different cell layers of the root can be worked out. Such microscopic measurements of piliferous layer cells and of cells of the outer cortex can easily be made on the intact root. Various workers have constructed mean growth curves by these techniques (*Figure 1.8*) and used them to record the influence of environmental and nutritive factors on the cell expansion process.

The dividing (meristematic) cells of roots are isodiametric, small, have a high nucleus to cell volume ratio and are filled with protoplasm. During

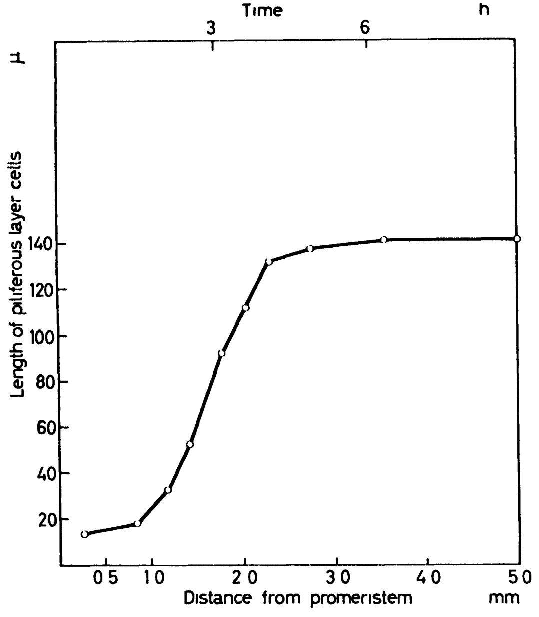


Figure 1.8. Curve showing the course of elongation of the piliferous layer cells of excised tomato roots growing in a culture medium containing 1·5 per cent sucrose[36]

cell expansion, not only is the cell volume increased dramatically, but the cells change in shape and develop easily visible vacuoles. Brown and his co-workers have now shown that root tip cells can be easily separated to give a cell suspension. This is prepared by a controlled maceration with chromic acid which breaks down the cementing substances between the cells (the middle lamella) but does not attack their cellulose walls. Further, the morphological differences between meristematic and vacuolated cells enable these to be readily distinguished in the cell suspension prepared with chromic acid. The numbers of each kind of cell per unit volume of the suspension can then be determined using a special microscopic slide first developed for counting blood cells. This slide is accurately ruled in squares of known area and a drop of the suspension mounted on it is spread out to a film of known thickness by the cover slip. The number of cells under each

square is counted microscopically (*Figure 1.9*) and from these counts can be calculated the number of cells per unit volume of suspension and the total number of cells in the root tip piece. This technique not only enables us to follow the number of new cells formed in the root tip during growth but also to observe changes in the number of meristematic cells or to check the constancy of this number.

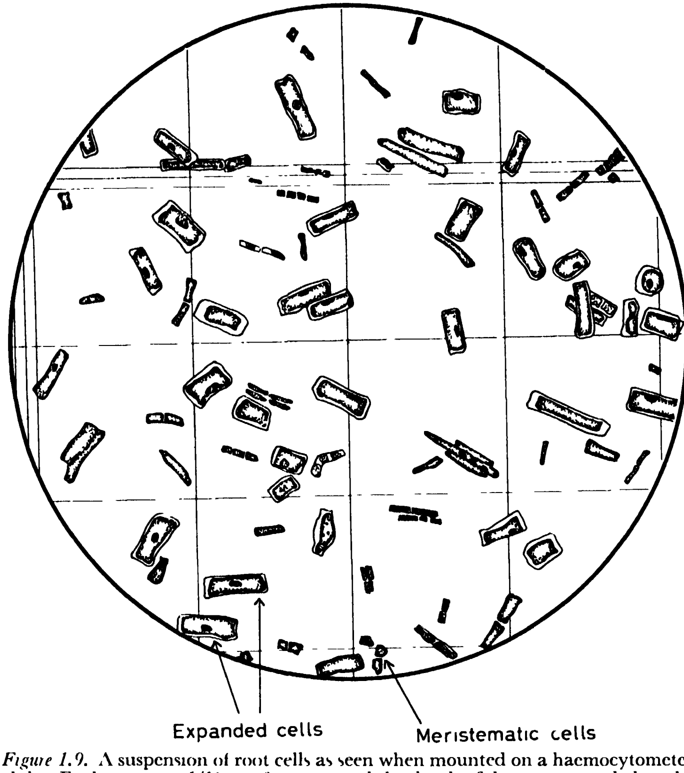


Figure 1.9. A suspension of root cells as seen when mounted on a haemocytometer slide. Each square is 1/16 mm² in area and the depth of the suspension below the cover slip is 0·2 mm.
(Drawn by Dr. D. N. Butcher)

When this information is added to knowledge of the rate of growth in length and the average change in size occurring during cell expansion we can study, under different chosen conditions, the contributions of cell division and cell expansion to the growth process. From knowledge of the number of meristematic cells and the number of vacuolated cells they have produced in unit time we can work out an average value for the time interval separating successive cell divisions in the mother cells, that is, the frequency with which each meristematic cell divides. Further, if in such suspensions, appropriately fixed and stained, we count the proportion of meristematic cells showing nuclear division we can work out the duration of mitosis.

Then using carefully prepared sections in which we can list the relative numbers of cells whose nuclei are at a particular stage of mitosis (prophase, metaphase, anaphase) the duration of each phase of the division process can be calculated. Such techniques open up the possibility of studying in considerable detail the influence of environmental and nutritive conditions and of physiological treatments on the processes of cell division and cell expansion in the growing apices of roots.

The visible changes occurring during the transformation of a newly initiated cell into a mature tissue cell are accompanied by characteristic physiological changes. The study of these physiological changes in a growing point such as that at the root tip requires methods capable of measuring physiological processes and determining chemical composition in minute amounts of living tissue. Special 'staining' methods have been evolved to determine the amount and location in cells of particular substances and in some cases of enzyme activity; these are the new methods of cytochemistry[37]. The *in situ* estimation of certain substances such as the nucleic acids, which are vitally important nuclear constituents, has been achieved by methods based upon their characteristic absorption of ultra-violet radiation of known wave-length. The electron microscope enables us to study changes in the minute structure of cell walls and of such important protoplasmic inclusions as plastids (centres of starch formation) and mitochondria (centres of the oxidation reactions involved in respiration). Micro-methods of estimating respiration, of determining proteins, carbohydrates and other important classes of cell constituents, and of estimating the activity of enzymes stable to extraction have also proved particularly valuable in studying the changes occurring during the expansion and maturation of plant cells. The techniques described earlier for counting cells in small masses of tissue have enabled the results obtained by these methods to be expressed not only on a weight basis but also in amounts per cell.

The process of mitosis is of short duration compared with the interval (interphase) between successive mitoses in the root meristem. Thus, for instance, Brown[38] working with seedling pea roots at 15°C recorded the following values: interphase 23 hours; prophase 2 hours; metaphase 25 minutes; anaphase 5 minutes, and telophase 22 minutes. Rise in temperature towards the growth optimum does speed up the mitotic process but the enhancement of cell division is primarily due to the shortening of interphase. This is not perhaps surprising, for it is during interphase that the reduplication of the chromosome material takes place: microchemical methods readily demonstrate the doubling of the deoxyribonucleic acid of the nucleus (DNA) during interphase. Further, although there is a marked rise in respiration immediately before the prophase of mitosis, the rate of respiration is very low during the stages of mitosis. The dividing nucleus is probably isolated from the centres of cell respiration and contains all the energy it requires from previous interphase activity.

The mature cortical cells of the root may have 20 or more times the volume of the meristematic initial cells from which they are derived. The process of cell enlargement which accounts for this is associated with the uptake of water leading to the appearance firstly of a number of small vacuoles and ultimately of a single central vacuole. This uptake of water, which is a

consequence rather than the cause of the cell expansion, is accompanied by large increases in cell dry weight, protein content and cell wall material. Simultaneously, characteristic changes occur in the physiological activity of the cell. The expansion process involves real growth and some degree of differentiation (change towards specialized function). The time course of this growth follows a characteristic pattern (*Figure 1.8*); at first growth is slow, then it accelerates to reach and maintain a high velocity until again, as the growth process reaches its completion, the rate quickly declines to zero. Studies on root hair development emphasize that cells have a limited expansion potential. The longest cells of the piliferous layer are either without hairs or produce the shortest hairs, while the shortest piliferous layer cells produce the longest hairs. Each piliferous layer cell appears to have a certain capacity for growth which may be expressed in either a longitudinal or horizontal direction. Studies on the wounding reactions of plant tissues and on the induction of cell divisions in fragments of mature living tissue by the techniques of tissue culture, however, emphasize the reversibility of cellular differentiation. We do not yet understand what factors determine the phases of the expansion and differentiation process or which can effect the return to the meristematic state. To begin to answer these questions we need to be able to describe the physiological changes which accompany changes in form and current research is being devoted to this task.

Brown and his associates, using serial segments of pea and bean roots, have revealed a changing pattern of enzyme activity during the process of cell expansion[39]. Not only are there significant changes in the relative activity of certain enzymes per cell but also per unit of cellular protein. Further, it has been shown that although during the process of expansion the synthesis of new protein and of new protoplasmic nucleic acid run parallel, the composition of the nucleic acid changes; the proportion of purine to pyrimidine bases in the nucleic acid rises as the cell matures. The changing enzyme pattern reflects this change in the protein composition of the cell. There are, also, changes in the respiratory activity both per cell and per unit of protein and changes in the sensitivity of the respiration to inhibitors. In pea roots, that fraction of the total oxygen uptake which is insensitive to inhibition by cyanide becomes a more important part of the total respiration during cell expansion and differentiation. James has obtained evidence that the enzyme, cytochrome oxidase, which is very active in the embryonic root of barley, is replaced as the principal enzyme mediating oxygen uptake by ascorbic acid oxidase as the proportion of mature tissue increases during radicle growth.

The process of cell expansion obviously leads to an increase in cell wall surface. The expansion phase in the growth of root cells always involves an increase in cell wall material usually to such an extent that the wall retains its original thickness during the period of most rapid increase in area and later increases in thickness. The cell walls of meristematic cells have, compared with the walls of differentiated cells, a relatively low content of cellulose (the carbohydrate which forms the wall's structural framework). The cellulose probably accounts for some 9 to 14 per cent of the wall volume. The low content of cellulose is associated with a relatively high content of

amorphous non-cellulose material (particularly hemicelluloses and pectins associated with smaller amounts of lipids and proteins). The content of water in the wall is probably not less than 60 per cent. Examination of cell walls by x-ray diffraction and the electron microscope indicates that the cellulose molecules of the cell wall are arranged in long bundles or microfibrils, and that these are the sub-microscopic threads from which the structural framework of the wall is woven. The adhesion of the microfibrils in this network is probably due to the development of weak chemical linkages (hydrogen bonds) between their surface-located chemical groups (*e.g.* free hydroxyl groups). Cell wall growth, therefore, involves expansion of this cellulose framework and the secretion into this framework of the non-cellulose constituents. Cell wall growth in certain cells is highly localized; the apical growth of root hair cells is a good example of this. However, studies of the incorporation of radioactive carbon have shown that cell expansion, in the region of linear growth immediately behind the root tip meristem, involves generalized growth of the surface of the cell walls.

As early as 1846 Naegeli put forward the idea of cell wall growth by intussusception, *i.e.* by the introduction of new materials *between* those that are already present. The other possibility, advocated strongly by Strasburger in 1882, is that cell walls grow by apposition, *i.e.* by the addition of material upon, not into, the existing wall. Whether both or only one of these processes is involved in the increase in cell wall area has proved difficult to decide despite our greatly increased knowledge of the structure of the walls of meristematic and of expanding cells derived from recent studies with the electron microscope.

The cellulose microfibrils of the walls of the meristematic cells of roots are predominantly transversely orientated relative to the root axis. During cell expansion this transverse orientation is not lost although it becomes restricted to the inner part of the wall, the outer part of the wall showing increasingly oblique or vertical orientation of its microfibrils (*Figure 1.10*). In keeping with this structure there has been advanced the 'multinet' theory of wall growth[41]. This postulates that although 'intussusception' into the existing wall, particularly of non-cellulose material, may occur to a limited extent, the main addition of new cell wall material, during cell expansion, occurs by 'apposition' against the inner wall surface. During growth each successive layer of microfibrils is therefore shifted outwards, stretched, particularly in the direction of cell elongation and its thickness and density of microfibril texture decreased. Such a concept invites comparison between wall structure and a set of superimposed fishing nets, successively stretched in the same direction. Hence the descriptive term 'multinet structure'.

Perhaps the most interesting question immediately posed by this theory is that of why the microfibrils are transversely orientated at the time of their deposition. Since protoplasm is the seat of synthesis of cell wall constituents it has been suggested that the microfibril pattern in the wall is determined by a pattern in the adjacent protoplasm. Roelofsen[41], however, has argued that the orientation of the microfibrils is primarily determined by tensions in the wall itself, the microfibrils orientating themselves in the direction of the strongest component of the wall tension induced by turgor. In cylindrical or near cylindrical cells the transverse tension will be dominant on the

inner face of the wall and the inner layers will bear the brunt of this tension component. The axial tension will, however, be relatively greater towards the outer wall surface and its dominance in the outer layers of microfibrils will tend to re-orientate them more and more in the axial direction.

The presence of protein in the walls of meristematic and young expanded cells and the evidence for protoplasmic continuity in living tissues suggests that the expanding wall may be generally permeated by protoplasm. The electron microscope evidence for this view is, however, controversial although

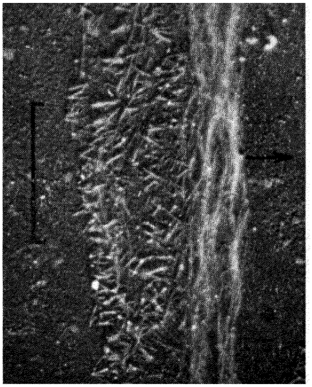


Figure 1.10. Electron microscope photograph of a transverse section through the outer wall of a piliferous layer cell of an onion root; the cell was situated about 1 mm from the tip[40]. The arrow points towards the centre of the cell. The scale corresponds to 1 μ

it does strongly suggest that microfibrils as such rather than isolated cellulose molecules are formed directly at sites of synthesis in the protoplasm. Not only are we uncertain of the site of cell wall synthesis but we are ignorant of the biochemistry of the synthesis of the main structural component, cellulose. Advances in our knowledge of the biochemical reactions involved in cell wall synthesis would greatly facilitate studies of the mode of action of the growth hormones whose controlling influence on cell growth is discussed in the next section.

Hormonal Control of Root Growth

Roots respond positively to the stimulus of gravity: when main roots are displaced from the vertical they bend to direct their growing tips towards the centre of the earth. This geotropic bending of the root is due to an unequal growth of the lower and upper sides of the root, particularly in the region behind the promeristem where the most active linear extension occurs. The pioneer work of Charles Darwin (1880) established that the stimulus of gravity was perceived in the root tip and transmitted to the growing region behind. Provided the tip was exposed to the unilateral action of gravity for

a minimum period (the reaction time) then a growth curvature resulted irrespective of the subsequent orientation of the root in relation to gravity. The subsequent studies of geotropism by the Russian plant physiologist, Cholodny from 1924 onwards, and the contemporary work of Went on the responses of the oat coleoptile to light and gravity, led to a general hormonal theory of tropisms. The outline of this theory as far as geotropism is concerned is as follows: The root tip produces a growth hormone or auxin similar to, or identical with, that secreted by the coleoptile of grasses. The concentration of the auxin thereby established in the growing cells is below the optimum concentration for expansion of the coleoptile cells but above the optimum for expansion of the root cells. The unilateral action of gravity diverts auxin to the lower side of the organ. In the coleoptile this causes the upper side to grow more slowly. The coleoptile curves upwards directing its tip away from the centre of the earth. In the root the exact reverse occurs and the root bends downwards. The important postulates of this theory as applied to the root are, therefore:

(*a*) The root tip secretes an auxin which controls the expansion growth of root cells.

(*b*) The growing root cells as a result of this secretion contain auxin at a concentration above the optimum concentration for their growth (at a supra-optimal concentration) so that cell expansion will be promoted by a fall or inhibited by an increase in this normal concentration.

(*c*) The unilateral action of gravity is perceived in the root tip.

(*d*) The tip, following stimulation by gravity, supplies less auxin to the cells in the upper part of the root and more to those in the lower part.

(*e*) It is this uneven distribution of auxin within the region of expanding cells which causes less growth to occur towards the lower and more towards the upper surface of the root and produces the downward root curvature.

This Cholodny-Went hypothesis was supported by Cholodny's contention that the immediate effect of removing the root tip was to cause a temporary enhancement of the straight growth of the root; presumably, cutting off the source of auxin caused the auxin concentration in the region of cell expansion to decrease and for a time to become more nearly optimal for growth promotion. Geotropically stimulated root tips placed upon decapitated but unstimulated roots induced curvatures pointing to transmission of the stimulus across the surface of contact by diffusion of some chemical growth regulator. Roots could be shown to contain material active in Went's standard oat coleoptile test for auxins and isolated root tips secreted auxin at the cut surface which could be collected by diffusion into agar jelly, particularly if the root tips were maintained physiologically active by receiving a supply of sugar from the agar. It has, however, proved difficult to establish that root cells obey Went's dictum 'no auxin, no growth' or that the curvature of roots in response to gravity is due to uneven distribution of auxin in the region of the root where cell expansion proceeds. It is from attempts to establish these postulates and from the use of the paper chromatography technique to examine the natural growth regulators of roots that our present, and still very unsatisfactory, knowledge of the hormonal control of root growth has been obtained.

The availability, from 1933 onwards, of β-indolylacetic acid (IAA) (*VIII*) (p. 38), the demonstration of its auxin activity in the standard auxin tests and the evidence pointing to its identity with the natural auxin of coleoptiles and shoots ushered in a period ofvoluminous research. Whole plants, plant organs and isolated plant tissues and organ segments were used in detailed studies of the physiological activity of IAA and of an increasing number of active synthetic compounds more or less related to it chemically. Such physiological studies with seedling roots and, more recently, with

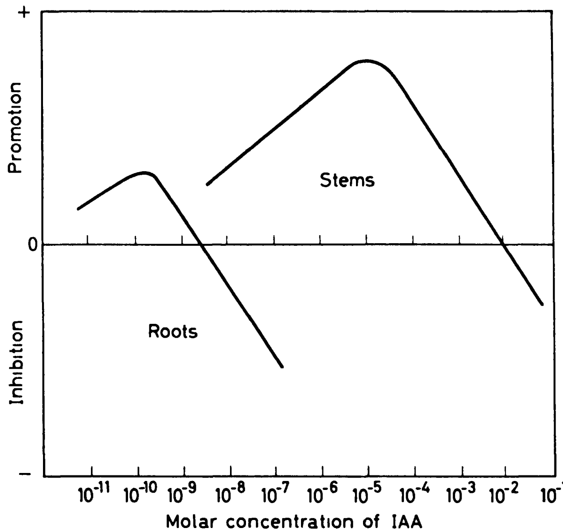
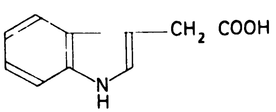


Figure 1.11. Growth responses to auxins of roots and stems[43]

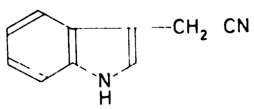
excised root cultures[42] have assessed the influence of IAA and other auxins not only on the expansion of root cells but on cell division, tissue differentiation and certain other aspects of root physiology.

Thimann[52], after carefully reviewing research published prior to 1937, concluded that the growth response of roots to auxin shows an optimum curve which parallels that of the growth response of shoots but with the optimum growth stimulation of roots occurring at a much lower auxin concentration (*Figure 1.11*). Subsequent work with seedling roots has amply confirmed the sensitivity of the roots to inhibition by auxin. Attempts to demonstrate the stimulation of root extension growth by appropriately low auxin concentrations have, however, yielded results whose interpretation is controversial. Thus, Torrey[44] in 1956 contended that: 'there seems to be little doubt that under many different conditions and in different plant species real stimulation may occur', whereas in 1957 Åberg[45] wrote: 'direct stimulation of the longitudinal growth of seedling roots by low auxin concentrations has never been convincingly demonstrated as a regular and reproducible phenomenon'. Important observations which probably go some way towards explaining the differing responses reported by different

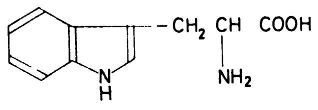
laboratories have been made by the Swiss botanist, Pilet. He found that the response of the root to external auxin changes as the root grows, very young radicles being both more readily stimulated by lower concentrations and less readily inhibited by higher concentrations of auxin than are older roots. Such changes in response were correlated with evidence that the internal auxin level in the root rises during growth, being initially below and later above the optimum value for root cell extension. If, therefore, auxin is essential for the expansion of root cells but if, as seems normally to be the case, secretion of auxin by the root tip establishes in the region of cell expansion a concentration above the optimum, then isolated segments of the expanding zone freed from the tip should theoretically be dependent upon external auxin for their continuing linear growth. Some workers have obtained rather convincing evidence that this is so. Audus and his co-workers[34], for instance, have described a marked enhancement of the elongation of 2-mm segments of seedling pea roots by very low concentrations of IAA.



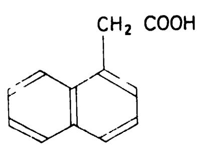
(VIII) β-Indolylacetic acid (IAA)



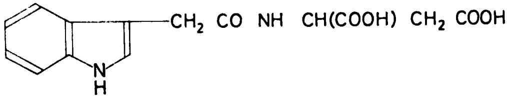
(IX) β-Indolylacetonitrile (IAN)



(X) Tryptophan



(XI) 1-Naphthaleneacetic acid (NAA)



(XII) Indolylacetylaspartic acid

The value of excised root cultures in such studies arises firstly from the more rigorous standardization of the environmental and nutritive conditions which they make possible. Secondly, for a number of species, the individual test root cultures can all be derived from a single initial root tip (the experimental material is a clone). This eliminates the genetic variability inevitably encountered when working with a population of seedlings and enables

readily reproducible responses to be obtained. This situation is well illustrated by recent detailed studies of the effects of IAA, of the related natural auxin, β-indolylacetonitrile (IAN) and of the synthetic auxin, naphthaleneacetic acid (NAA) (*IX* and *XI*) upon the growth and differentiation of excised tomato roots. In standard root culture medium and with concentrations above 10^{-10} g/ml. of IAA, above 10^{-7} g/ml. of IAN or above 10^{-10} g/ml. of NAA, the growth of excised tomato roots is inhibited[40]. Lower concentrations of these auxins do not significantly enhance growth. However, if the sugar concentration (2 per cent) of the standard culture medium is reduced to 1 per cent or lower then appropriately low concentrations of either IAA or NAA induce very significant enhancements of root growth. Studies of these growth promotions show that both cell expansion and cell division are stimulated. The relationship exposed here between carbohydrate status and the response to auxins cannot at present be explained but its importance will be further emphasized in discussing factors which control the duration of activity of root meristems.

When IAA or NAA induce growth inhibitions of the order of 50 per cent they do so by reducing mean cell length and not by inhibiting cell divisions at the meristem. The duration of the process of cell expansion is not affected but the longitudinal component of this expansion is reduced. In certain tissues, notably the cortex. there is a compensating increase in the transverse areas of the cells as seen in section so that root diameter is increased. Further, such reductions of linear growth are not paralleled by a corresponding decrease in total dry weight and the percentage dry weight is consistently increased. With concentrations of these auxins causing increasingly higher degrees of inhibition the reductions in average cell length less and less account for the over-all reductions in root extension growth. This discrepancy is, at first, due mainly to altered polarity at the meristem so that relatively more longitudinal and relatively fewer transverse division walls occur, thereby increasing the number of cells in the transverse section of the root. This is well reflected in an increased number of xylem vessels in the transverse section and a smaller number of vessel units per unit length of each vessel. At still higher concentrations these auxins inhibit cell division. In contrast to the inhibition of root growth by IAA, that due to IAN is over the whole range of inhibition due mainly to a reduction in division rate at the meristem. IAN is a very weak inhibitor of cell extension. Further, in excised tomato root, IAA does not markedly influence lateral root initiation although at appropriately high concentration it does act as an inhibitor of lateral emergence and extension. IAN, although characteristically inhibiting the cell division rate of the main axis tip meristem, does significantly enhance lateral initiation and, at sufficiently high concentration, induces some secondary increase in the root conducting tissues.

In roots of other species and under appropriate experimental conditions IAA has, however, also been shown to enhance lateral initiation. Even more marked effects are often to be observed when studying the initiation of adventitious roots on stem cuttings. The influence of externally fed auxins on the initiation of laterals is probably dependent upon the levels of internal auxins and of other essential factors. Evidence has been obtained that factors such as carbohydrate supply, biotin, thiamine, adenine and the

hypothetical 'rhizocaline' (a root growth hormone synthesized by the shoot in light) are involved along with auxins in lateral initiation.

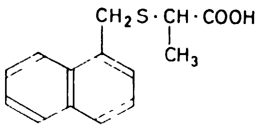
Reference has already been made to the increase in percentage dry matter associated with the application of partially inhibitory concentrations of auxins to excised tomato root cultures. Anatomical studies show enhanced wall thickening and enhanced lignification of the xylem conducting cells of inhibited roots. Clearly, auxins affect not only cell division and expansion but also tissue differentiation. Similar observations come also from studies with root cultures of other species. Torrey, for instance, working with pea root cultures has shown that whereas the 'uncoupling' reagent, dinitrophenol, inhibits both root growth and differentiation of the conducting tissues, IAA at inhibitory concentrations markedly accelerates the differentiation of the xylem. Studies with herbaceous shoots and callus cultures also clearly indicate that the establishment of a sufficient auxin concentration and its transmission along a pathway in the tissue are among the determinative factors initiating vascular strands and particularly the differentiation in them of xylem.

The continuous, interrupted or limited duration of function of root meristems, their level of cell division activity and the inhibitory influence of the active meristems on the functioning of adjacent and more recently initiated lateral meristems together determine the general forms of the root systems of plants. Thus, in some plants there is, as already indicated, a dominant, continuously growing and prominent tap root; in others we find a number of equally prominent roots each of which completes its growth in a fraction of the plant's life-history. Studies of the form and development of root systems emphasize that root meristems may not only show seasonal fluctuations in their cell division activity but that some have a limited duration of activity even under favourable environmental and nutritive conditions. Such meristems have a 'grand period' of growth which seems to involve a phase during which the meristem develops its full size and activity, and at some interval after this a phase of declining activity leading ultimately to the cessation of cell division and even loss of the organization and meristematic character of the root apex. In this latter phase the meristem can be regarded as senescent.

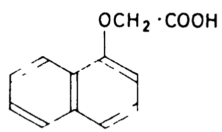
We have referred earlier to the excised roots of certain species as having been established in continuous culture. It is, therefore, of interest to enquire whether this means that the functional life of their apical meristems has been indefinitely prolonged. Clones of excised tomato roots have probably been maintained in culture for the longest periods. White maintained some of his original clones for at least 3 years. In our own laboratory, clones have been maintained for as long as 5 years and in all cases excised tomato root clones have maintained their characteristic growth rates over long periods terminating in every case either by being voluntarily abandoned or by becoming accidentally contaminated with micro-organisms. However, these clones are maintained and the amount of clonal material multiplied by the use of 'sector cultures', cultures initiated from short pieces of the main root axis bearing five or six young lateral roots (*Figure 1.6*). Newly initiated lateral meristems carry on the growth of the clone at each subculture. When the same apical meristem is each time excised and used to initiate the next

culture passage in White's standard root culture medium it becomes clear that each individual meristem is only capable of a limited period of growth. Using a particular clone, 10-mm root tips for subculture and growing the cultures each time for 7 days, it was found that nearly all the meristems had become quite inactive in 7 weeks[46]. Anatomical examination showed cell differentiation right up to the region of the promeristem and the promeristem cells contained numerous small vacuoles. When individual excised root tips were grown, without subculture, in flowing sterile culture solution it was found that within 21 to 28 days the initial apical meristem had ceased to function. The subsequent growth of the cultures reflected the activity first of their primary lateral meristems and, when these in turn ceased to function, of the activity of the meristems of the second-order laterals. Further, under these conditions involving the development of large root systems, the duration of function of the initial meristems and the lengths of main root axis to which they gave rise were less than when the meristems were excised every 7 days.

Some further prolongation of the average functional life of the meristems could be achieved by transferring at each subculture 5-mm rather than 10-mm tips and by reducing each growth period from 7 to 3 days. The functional life of the meristems was reduced by some influence emanating from the mature root tissue and its associated secondary meristems.



(XIII) α-(1-Naphthylmethylsulphide) propionic acid (NMSP)



(XIV) 1-Naphthoxyacetic acid (1-NOA)

In the culture period(s) immediately preceding the cessation of meristem function the roots become abnormally thickened, yellow and contorted resembling, in both form and anatomical detail, roots inhibited by auxins or cultured in presence of high concentrations of sucrose. This suggests that some auxin (or its precursor) may be accumulating in the root first to a concentration reducing the rate of cell division and later to a critical concentration at which the cells of the meristem differentiate and lose their capacity to divide. In support of this hypothesis it was shown that externally applied NAA or IAA, even at low concentrations, reduced the duration of activity in the meristems. By contrast, the duration of activity was dramatically enhanced by using substances (anti-auxins such as α-(1-naphthylmethylsulphide) propionic acid and 1-naphthoxyacetic acid (*XIII* and *XIV*)), known to antagonize auxin action. When cultures were allowed to develop without subculture these anti-auxins prolonged active growth of the main axis and intensified the natural apical dominance (*Figure 1.12*). Reduction of the sucrose concentration of the culture medium to 0·75 per cent or lower was even more effective than the anti-auxins in prolonging the duration of

activity of the meristems, again suggesting that carbohydrate supply controls auxin synthesis or the expression of auxin activity.

Studies of a similar 'ageing' phenomenon in excised red clover roots[47] strongly point to the level of some natural auxin being only one of the

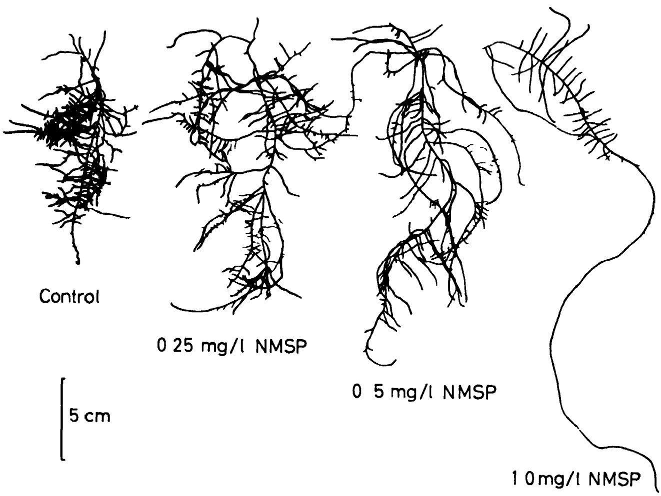
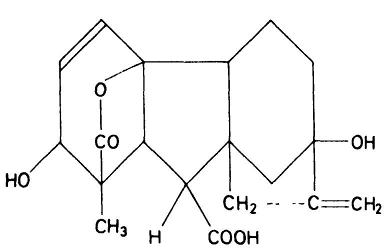
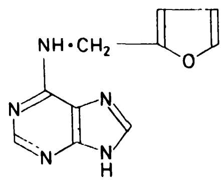


Figure 1.12. Excised tomato roots grown for 31 days in standard medium (control) and in this medium with the addition of the anti-auxin NMSP (*XIII*) at the concentrations indicated[46]. Note the increasing apical dominance of the main axis and of the first-order lateral roots with the higher concentrations of NMSP

factors involved in the control of cell division in root meristems. Similarly, the recent observations in work with tomato roots, that gibberellic acid (*XV*) can also, like auxin, shorten the duration of meristematic activity and that



(XV) Gibberellic acid



(XVI) Kinetin (6–furfurylaminopurine)

the cell division factor, kinetin (*XVI*), can antagonize the deleterious effect of auxin or high sugar concentration also point in this direction. Gibberellins are natural constituents of excised tomato roots. Substances chemically very similar to kinetin are natural cell constituents although neither kinetin nor an active derivative of it have yet been isolated from plants. Nevertheless, the possibility clearly exists that the balance between cell division and cell

differentiation in the growing points of roots may be controlled by a balance between a number of natural growth factors, of which a natural auxin was the one whose concentration rose to a critical level in the studies with excised tomato roots outlined above.

From the account presented it appears, therefore, that under the standard conditions usually adopted in root culture, excised tomato roots quickly develop a supra-optimal concentration of auxin so that the physiological activity of externally applied auxins is expressed in a growth inhibition. By contrast, excised roots of Petkus II rye can only be maintained continuously in culture by supplying in the medium either IAA or the auxin-precursor, tryptophan (*X*). The same seems to be true for roots of the pea variety World's Record. There are, however, a number of cases where excised root growth is not absolutely dependent upon an external supply of auxin, but in which auxin, at an appropriate concentration, is significantly stimulatory to growth. The growth of excised roots of pine, white lupin, and of varieties of pea, maize and wheat is stimulated by auxins and in some studies with these roots evidence has been advanced that the auxin activity present in the seedling radicle declines markedly during growth in culture, leading, in the absence of an appropriate external supply, to the development of an auxin deficiency. Work undertaken by Charles[48] on four geographic strains of common groundsel strongly indicates that the different behaviour of their excised roots in culture is a reflection of their differing and in each case, sub-optimal contents of a natural auxin.

Reference has been made earlier to the resistance to culture of the excised roots of monocotyledons and particularly of roots from cereals since it is these which have been most intensively studied[49]. The only successful continuous cultures of cereal roots are those of Petkus II rye[50]. In this instance, successful culture depended upon the adoption of a suitable subculture programme and the incorporation into the culture solution of autoclaved tryptophan or of IAA. However, this technique does not solve the general problem of the continuous culture of other cereal roots or even of other varieties of rye or even of the root tips from all seedlings of Petkus II rye.

Recent work with excised roots of Atson wheat has shown that the addition of casein hydrolysate to the medium together with illumination of the cultures makes possible many successive subcultures. There is, however, a marked decline in growth rate after about 14 days in culture and subsequently a slower decline and ultimate cessation of growth. The addition to the culture medium of autoclaved tryptophan, at concentrations inhibitory to 'light' grown cultures, markedly enhances the growth of cultures incubated in the dark. The ability of autoclaved tryptophan to promote continuing growth of dark incubated roots is, however, inferior to that of appropriate illumination in absence of tryptophan. A similar beneficial effect of light on the growth of excised roots of maize and other species has previously been reported and a critical examination of published work does not exclude the possibility that some light is necessary for the growth of all root cultures, a view which gains credence from the very low light intensities which can be shown to promote the growth of wheat roots. As long ago as 1933, Went and Bouillene postulated a hormone, 'rhizocaline' synthesized in the shoot

and functioning as an essential root growth factor. This hormone has remained hypothetical but has been invoked by a number of workers to explain their experimental observations. Root cultures may also have a requirement for this 'rhizocaline' and be required to be exposed to light to effect a photochemical stage in its synthesis. It could also be that in some roots, such as those of rye, a product arising from tryptophan during autoclaving can act as an effective precursor for this essential hormone in the absence of light.

The picture which seems to emerge from these physiological studies is that of a number of hormonal factors regulating root growth. Auxin, or to be more specific, IAA would then be regarded as one hormone of this group and its physiological activity would, in consequence, depend not only upon its own concentration but upon the levels of other essential growth factors simultaneously present and equally involved in the control of growth and differentiation. Clearly, to obtain direct evidence of a growth-regulating mechanism such as is now postulated it is essential to detect and ultimately to identify the natural growth regulators present in root cells. It is, therefore, appropriate to outline some recent studies in which growth-active substances in root extracts have been separated by paper chromatography.

Paper chromatography separates chemical compounds by virtue of their different rates of movement in a current of water-containing organic liquid (the 'solvent') flowing within a strip of filter paper. The filter paper strip, subsequently dried and freed from the 'solvent', constitutes the chromatogram. The location of compounds on such a chromatogram can be ascertained by examination in ultra-violet light (detection of fluorescent compounds), by spraying with reagents giving coloured derivatives with the type of compound sought or by testing the biological activity which can be washed off sequential segments along the length of the strip. Further, when chromatographed, each particular compound moves a fraction of the distance moved by the 'solvent' front and such fractions, known as R_f values (for instance, 0·25 means 1/4 of the length of the chromatogram) are recorded in the literature from work with pure compounds and specified solvents. Such R_f values may be a very important guide to the identity of an unknown compound and can also be quoted to characterize growth-active regions of the chromatogram where chemical identity has yet to be established.

Such chromatographic separations of the auxins in plant extracts were first successfully carried out by Bennet-Clark and his co-workers at King's College, London, and have formed the subject of papers published by this and other laboratories since 1952. In such studies, the distribution of auxins along the chromatograms has been followed by determining the ability of the sequential pieces of the chromatogram to yield material promoting the growth in length of measured segments cut from young grass coleoptiles. Auxin activity on chromatograms of root extracts has, therefore, in most studies so far published, been determined by this test which measures a promotion of the growth of shoot cells.

Plant tissues to be examined for auxins are first extracted at or below freezing point with ethyl or methyl alcohol, the alcohol removed and the aqueous residue then successively shaken with ether or ethyl acetate. Under

these conditions the known plant auxins such as IAA and the corresponding β-indolylacetonitrile (IAN) pass almost entirely into the ether and it is such ether extracts which have, in general, been submitted to chromatography. Work with root extracts has now shown that they contain such ether-soluble auxins at very low concentration[42]. However, by loading sufficient extract on to the chromatogram the presence of at least four separate growth-active regions can be demonstrated. The activity of one of these regions does seem to be due to IAA, although it is always present at too low a concentration to give a specific colour reaction. The chemical nature of the other zones is unknown. One is always a region of growth inhibition and one of the growth-promoting zones clearly contains more than one active compound.

However, in these studies on root extracts it has been found that the greater part of the auxin activity does not pass from aqueous solution into ether; most of the auxin activity extracted from roots is preferentially water-soluble. Very active chromatograms of the 'aqueous fraction' can be obtained with relatively very low levels of loading and such chromatograms show that a number of compounds contribute to this activity. The active regions give colour reactions with the amino acid reagent, ninhydrin. However, the R_f values recorded indicate that while the amino acid, tryptophan, is one of the active compounds, the others do not correspond to any of the common amino acids.

Clearly, this work on the chemical identification of the auxins present in roots is in its infancy. It does, however, emphasize that we are concerned not with *an* auxin but with a complex of substances active as auxins in low concentration. This concept that the auxin content of roots is altogether more complex than hitherto envisaged is also emphasized by recent experiments involving chromatography of extracts taken from growing root cultures fed, under controlled conditions, with known auxins such as IAA and IAN. For instance, feeding with IAA not only leads to accumulation of this compound in the root but to the rapid appearance of some five other indole compounds at concentrations which enable them to be located on chromatograms with colour reagents. Two of these indoles are active auxins and in one case the activity is due to a conjugate between IAA and the amino acid, aspartic acid (*XII*). The identity of the other indoles is uncertain but it can be suggested from current evidence that they include the conjugate of IAA and glutamic acid and also indolylacetamide. Furthermore, as soon as the roots are transferred back to an indole-free culture medium the concentration of these indoles in the roots quickly falls, suggesting that they are readily metabolized. These feeding experiments not only reveal the rapid formation and disappearance in root cells of indole compounds not normally detected on chromatography of root extracts but also reinforce earlier physiological evidence that the activity of IAN as an inhibitor of root growth is not due to its conversion to IAA within the root cells.

Recent work, using root cultures, has also shown the presence in roots not only of auxins but of substances with the same physiological activity as the fungal gibberellins. Suitably purified root extracts have been chromatographed and the distribution of gibberellin activity along the chromatograms followed by the highly specific test with the Meteor variety of dwarf pea, in

which application of gibberellin induces a tall habit by stimulating stem growth.

As indicated, growth-active compounds detected in root extracts have been so defined primarily by reference to their ability to promote the expansion of shoot cells. Clearly, in attempting to assess the role of these substances in the control of root growth, it is important to use a root growth test. The standardization of such a test using excised tomato root tips has recently been undertaken in our laboratory and its use has immediately revealed the presence in root extracts of powerful root growth inhibitors. This raises the important question of how far interactions between natural growth-promoting and growth-inhibiting substances are important in the mechanism of growth control. Since this section was introduced with an account of the Cholodny–Went hypothesis it may be interesting to refer to some recent work on geotropism which suggested the action of a growth inhibitor which was not IAA or any similar compound. Audus and Brownbridge[51] obtained evidence that the geotropic response of their test roots was due to the *de novo* production of an endogenous inhibitor in the extending cells on the lower side of the root, whence it later spread to the upper side. In the classical hypothesis the lower side of the root is thought to grow more slowly than normally, the upper side more quickly than normally. The interesting observation which prompted the studies of Audus and Brownbridge was that during geotropic curvature *both* sides of the root grew more slowly than normally.

The evidence that interactions between a number of natural hormonal factors control cell division, cell enlargement and the different pathways of cell differentiation leads us to ask what physiological processes determine these aspects of growth and what part the controlling factors play in these processes. In the case of those plant growth factors which also act as vitamins in animal nutrition, for example the vitamins of the B complex, we know that they act as essential parts (co-enzymes) of enzyme systems. We do not have any such understanding of how auxins and gibberellins exert their physiological activity.

It has been pointed out[53] that auxins operate only in plants with cellulose cell walls and that, therefore, their action may be mediated primarily through controlling cell wall growth. The effects of auxins on cell wall thickness and on lignification in fully expanded cells would also point in this direction. It is, however, difficult to explain the influence of auxins on cell division on this basis. This has led to the alternative hypothesis that auxins control some 'master reaction' which in its turn influences many aspects of metabolism. However, it would be going beyond our present subject of root physiology to discuss the large body of inconclusive experimental data relative to these two concepts. This, however, may perhaps be taken as illustrative of the very general significance of many of the problems encountered in the study of root physiology.

CONCLUSION

The scope of this chapter has been comprehensive in the sense that it has brought together a number of different aspects of the physiology of roots.

Inevitably, the treatment of each aspect is very incomplete both from reasons of space and from the limitations of our present understanding. Nevertheless, it is hoped that it may have succeeded in focusing the reader's attention upon some interrelationships not hitherto appreciated and that by indicating where present knowledge ends it may stimulate further researches in root physiology.

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HISTOCHEMISTRY IN BIOLOGY

P. R. LEWIS

INTRODUCTION

ALTHOUGH Science as a whole advances continuously (and at an ever-increasing rate) progress in a particular science may be erratic. Many examples could be quoted where technology is the rate-determining factor, some major advance in technique initiating a sudden but often brief re-awakening of interest in old, unsolved problems: such has happened more than once in the science of microscopy[1]. Occasionally, however, a subject may lie dormant because its true significance is not generally appreciated. Then, when interest is finally aroused, there may follow such a dramatic and sustained expansion of knowledge as to create what is virtually a new science. In just such a way has modern microscopical histochemistry evolved during the last two decades; but before we discuss the fascinating discoveries of the last twenty years it is important to examine the reasons why the subject became dormant almost half a century earlier.

Histochemistry, the study of the chemical make-up of tissues at the cellular level, became recognizable as a separate science towards the middle of last century and both Baker[2] and Pearse[3] regard Franzçois-Vincent Raspail (1794–1878), before he turned towards broader, political horizons, as its real founder. During the latter half of the century the new science continued to flourish and expand, although there was surprisingly little cross-fertilization, in ideas or techniques, between the botanists and the zoologists. Towards the end of this period there appeared a sharp division within animal histochemistry. The study of whole-organ chemistry followed the then new science of physiology and later, of course, became a science in its own right -biochemistry. But the microscopical study of cellular chemistry mostly remained with anatomy under the guise of histology and as such became a dormant or, at best, a quiescent science.

The decay of histology as a science can be traced to the introduction, by Bencke in 1852, of the aniline dyes which were in general use by 1880, followed closely by the development of paraffin-sectioning and photomicrography as routine techniques. Thus, by the end of the century, a fashion was set in histology which even today has not been completely supplanted. In retrospect it may seem strange that the attention of histologists was concentrated for so long upon descriptive morphology without their making any serious attempt to study the chemistry of the structures they were staining. The principal reason is probably to be found in the changing system of education whereby increasing specialization left the biological, particularly the medical, student less conversant with chemistry and physics than his predecessor. Nevertheless, there were plenty of chemically-inclined

biologists and biologically-inclined chemists ready and able to contribute when interest in histochemistry proper was finally re-awakened shortly before the Second World War.

It is difficult to pin-point the major cause for this re-awakening but certainly the subject was over-ripe for development, with many techniques devised by chemists and biochemists waiting to be applied to tissue sections. Two important factors were the development by biochemists of micro-analytical methods applicable to small homogeneous samples or even to single, large cells[4] and the publication of Lison's *Histochemie Animale*[5] in 1936. From then onwards more and more techniques were developed for the study of cellular chemistry. Although there has been a recent falling off in the rate of introduction of basically new techniques, the development and use of existing ones has continued to increase.

Histochemistry has now become so vast a subject that no attempt is made in the present article to review it all! Instead, a few techniques have been selected to illustrate the types of principles involved and the sort of information obtainable. Many topics have had to be omitted: very little is said about the important subjects of fixation and processing of tissues, and no attempt has been made to cover techniques involving complex physical apparatus unlikely to be available to the general biologist. Throughout the review there is a perhaps regrettable bias towards animal histochemistry—but many of the techniques can be and have been applied to plant tissues, although the technical problems encountered are rather different. Since it is the use of a staining procedure with known chemical specificity that distinguishes a histochemical from a histological technique some chemistry has necessarily been introduced but is sufficient only to explain the basis of the methods discussed. For a fuller account both of histochemical techniques and of their chemical basis the latest edition of Pearse's textbook[3] should be consulted.

The range of histochemistry is now so wide that it is difficult to give a simple classification of the types of techniques employed, but the following table will serve as a rough guide for the present review:

1. Physical methods
2. Chemical methods
    (a) Physical binding of chromophore group
    (b) Covalent binding of chromophore group
3. Biochemical methods
    (a) Production of stain by enzymic activity
    (b) Adsorption of fluorescent antibodies

The second group, with which we shall largely be concerned, can be further subdivided: the colour may reside entirely in the added chromophore group, in which case all the reacting tissue components will have the same colour or, alternatively, the compounds to which the chromophore becomes attached may, to some extent, determine the colours produced, thus allowing some distinctions to be made between the possible reacting substances. Most of these chemical tests depend upon the presence in the tissue component of a specific grouping, for example, an aldehyde group, and this may either be present in the naturally-occurring tissue or be produced by a suitable

chemical pretreatment such as oxidation. Techniques depending upon most of the possible combinations are given in this review. As is inevitable with such a simple classification, there are many techniques which overlap the subdivisions and some which are not included at all. Most of the techniques are not, in themselves, specific for one single substance but give a positive reaction with a whole class of chemically related compounds. There are several important methods of improving specificity· certain of the reactive compounds may be removed, for instance, by the action of specific enzymes, or rendered inactive by suitable chemical procedures, before applying the main histochemical test. Rather than discuss these auxiliary methods together but in isolation, I have introduced them individually wherever seemed most appropriate.

STAINING BY BASIC DYES

One of the most powerful techniques available to present-day histochemists is based upon the staining of acidic structures by basic dyes, that is, by coloured molecules possessing a positive charge. The essential linkage by which such dyes are bound is ionic. Although certain dyes have selective staining properties which they owe to the formation of additional bonds of a non-ionic character, their action still depends primarily upon the attraction of a positively charged molecule by negatively charged groups in the tissue section. We must, therefore, consider the types of acidic groups which may be present in tissue sections and the conditions under which they do or do not carry a net negative charge. Of those naturally occurring in tissues the only ones we need consider here are carboxylic, phosphoric and sulphuric acid groups. Typical substances in which these occur are, respectively, mucopolysaccharides, nucleic acids and chondroitin sulphate. The latter substance is so strongly acidic that it is always virtually fully ionized irrespective of the pH of the solution in which it is. This is not true of the other two. Most carboxylic acid groups have apparent pK values in the range of 4 to 5. Hence the proportion of such groups carrying a negative charge falls very rapidly as the pH is progressively lowered through this range. The intensity of staining with a basic dye falls off correspondingly. In effect, hydrogen ions and dye molecules compete for the available acidic groups, the balance being very dependent upon pH near the apparent pK of the acidic group. Similar considerations apply to the staining of phosphate groups, but here the critical range is somewhat lower. These facts have been made use of in a wide variety of techniques.

One specifically histochemical use of basic staining is the determination of the so-called extinction point or the pH at which a particular structure ceases to stain with a basic dye[6]. The principle of the method is to carry out the staining under conditions such that the intensity of staining is an accurate measure of the concentration of groups carrying a negative charge at the particular pH used. Normally, serial sections are stained in a series of dye solutions buffered to appropriate pH values for a time sufficient for equilibrium to be reached. Careful comparison of these sections then shows which structures have staining that is highly dependent upon pH over any particular range. For this technique, methylene blue is the basic dye of

choice because it is so free of any selective staining due to non-ionic bond formation. Since other cations besides hydrogen ions can compete with the dye, the staining solution should not contain large amounts of electrolytes; so one must use a dilute solution of methylene blue containing just sufficient buffer to give an adequately stable pH. Ideally, one should use the same buffer system for the whole pH range and the veronal acetate buffer of Michaelis is usually recommended. A formic acetate buffer system[7] is rather more satisfactory, however, over the pH range most commonly used. The stained sections should first be examined in water. For permanent preparations they are best mounted in balsam but differential extraction of the dye during dehydration must be avoided, preferably by the use of acetone in which methylene blue is only sparingly soluble.

This technique of controlled staining with methylene blue can be used for the study of a number of biologically-important compounds. Theoretically at least, it could be used in the study of proteins. Above its isoelectric point a protein molecule will carry a net negative charge and will stain with a basic dye: below its isoelectric point it will be positively charged and will stain faintly or not at all. Similarly, a protein should stain with suitable acidic dyes below its isoelectric point but not above. These theoretical deductions are borne out in practice[6], but the technique is of little use in protein histochemistry since the isoelectric points of most native proteins fall within a fairly narrow pH range, and their apparent isoelectric points in tissue sections are profoundly influenced by the fixative used. (For example, formalin fixation, which destroys many of the potential basic groups, causes most proteins to stain down to a lower pH than they do in the native state.) Thus, it is in the study of substances containing sulphate or phosphate groups that basic staining provides the most precise information.

The study of sulphonic acid or sulphuric acid groups presents no difficulties of specificity. If the staining solution is made sufficiently acid, these are the only groups which will stain with most basic dyes. The precise degree of acidity required to give absolute specificity is debatable. On theoretical grounds a pH of about unity (*e.g.* 0·1N hydrochloric acid) should be used, but a pH of 2·5 is low enough for most routine studies, provided that the possible faint staining of nucleic acids is remembered.

For the study of the nucleic acids and other substances containing phosphoric acid groups a compromise must be struck between a weakening of staining if the pH is too low and some staining of carboxyl groups if it is too high. In practice a pH of 3·5 to 3·8 gives reasonably selective staining of phosphate groups (although sulphate groups are still stained). This is a somewhat higher pH than might be expected from the measured dissociation constant of purified nucleic acid in free solution. The probable reason for this discrepancy is that in tissues the nucleic acids are normally associated with basic proteins.

When carboxyl groups are the only ionizable groups present, as in a pure mucopolysaccharide, staining becomes apparent at about pH 4·0 but when basic groups are also present, as in proteins and mucoproteins, a higher pH is needed. Thus basic staining cannot be used as a general method for carboxyl groups, but controlled staining with methylene blue is a valuable tool for distinguishing various types of polysaccharide derivatives

(*Plate 2.1(a)–(d)*). As with proteins, however, the extinction point for mucoproteins does depend upon the fixative used.

The competitive effect of other cations can be utilized to increase the specificity of basic staining. Thus nucleic acids are said not to stain if the solution is saturated with magnesium chloride. Similarly, aluminium ions prevent the staining of both phosphate and carboxyl groups, and this has been made the basis of a recent, very specific technique for sulphate groups[8].

A common method of improving the selectivity of basic staining is to use a dye which unlike methylene blue has a specific affinity for particular tissue components. One example, alcian blue, which has an affinity for mucopolysaccharides but not for nucleic acids, is mentioned later and many others can be found in any textbook of histochemistry. However, in no case is the exact chemical basis for the special affinity known; so their use in histochemical studies must always be carefully controlled. For certain problems, however, they are ideal for routine use provided occasional controls are made with fully acceptable histochemical techniques. Such selective dyes have long been used in histology but it is still not generally appreciated just how far they can be improved by the careful control of pH and other factors. An example is the use of cresyl fast violet as a stain for Nissl substance in the central nervous system. As used for many years this method consisted in gross overstaining in a strong, unbuffered solution of the dye and differentiation with the exercise of great care and artistry until, if ever, the desired result was obtained. By using a more dilute and carefully buffered solution of dye[7] one can obtain excellent, reproducible staining without any differentiation at all. The optimum pH depends upon the species and fixative used but need be discovered only once for each combination. Control of pH is particularly valuable where several dyes are used successively.

Metachromasia

No account of basic staining would be complete without some discussion of the phenomenon of metachromasia – the staining of some tissue components in a colour markedly different from the orthochromatic colour of the dye in aqueous solution. Although an effect of this type is observed with a number of dyes, here discussion can conveniently be restricted to the thiazine group which includes methylene blue, toluidine blue and thionine. Cartilage, for instance, stains an intense red or purple with these three dyes whereas many other structures stain blue. Such metachromasia has yet to be fully explained, particularly the precise conditions necessary for its occurrence. The chemistry underlying the actual colour change, however, is fairly well understood[9]. Briefly, all three dyes quoted have absorption maxima at the red end of the spectrum in dilute aqueous solution where they are present as individual molecules. In more concentrated solution some dimerization occurs; the two dye molecules are closely associated side by side with no water between them. Such dimers have absorption maxima at somewhat shorter wavelengths, thus transmitting some red light and appearing purple in solution. Higher polymers can also form, absorbing light of even shorter wavelengths and appearing almost red. Substances with a large number of

(a)

(b)

(c)

(d)

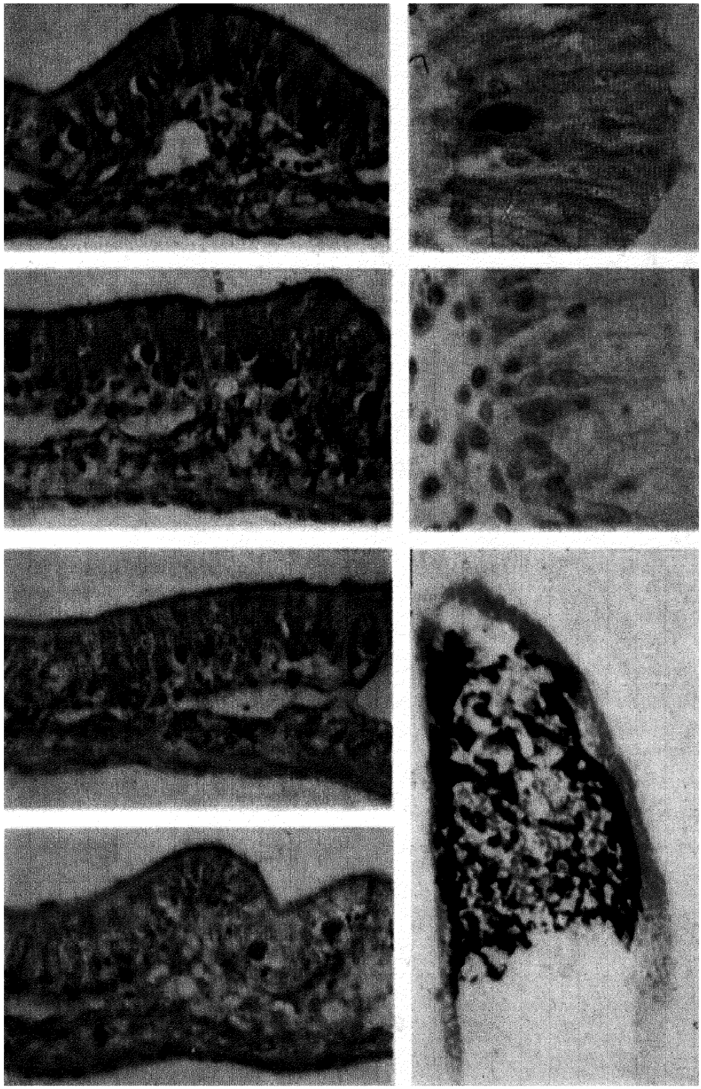


Plate 2.1. The left-hand side shows sections from adjacent slides of intestine from a *Xenopus* tadpole treated by the PAS technique and then stained with methylene blue at a closely-spaced series of pH values: (*a*) 4·9, (*b*) 4·5, (*c*) 4·1, (*d*) 3·7. Note the rapid change in staining of mucopolysaccharides over this pH range. The right-hand side shows, above, a pair of sections of rabbit intestine with individual enterochromaffin cells stained by: (*e*) the ferric ferricyanide technique, (*f*) coupling with diazonium salt (Red TR) and below, a section of a foetal mouse limb-bud (*g*) stained by the simultaneous coupling azo dye technique for alkaline phosphatase followed by the von Kossa silver technique for calcium deposits.

To face page 54

closely-spaced negative charges on their surface encourage this polymerization of the thiazine dye.

Since the acidic groups must be ionized to give any staining at all, metachromatic staining depends upon pH as does orthochromatic staining. Mere possession of acidic groups is not, however, a sufficient requirement. Pure proteins seldom, if ever, stain metachromatically. Mucopolysaccharides often do, although as the pH is lowered staining tends to become orthochromatic before disappearing entirely. Also, their metachromasia is often lost or diminished if the slides are dehydrated through alcohol. Dehydration through acetone is better but the results must still be interpreted with caution unless the sections can be examined in aqueous solution. Acid mucopolysaccharides, on the other hand, always exhibit strong metachromasia even after dehydration in alcohol. Usually, nucleic acids do not show marked metachromasia—either because the phosphate groups are so arranged as not to encourage dye polymerization or because their charge is partially neutralized by association with the basic groups of proteins. Because the conditions necessary for its occurrence are so obscure metachromasia is valuable only in the identification of acidic mucopolysaccharides and in certain specific histochemical techniques mentioned later.

Some Recent Methods Involving Selective Pretreatment

The range of usefulness of basic staining can be greatly extended by combining it with suitable specific pretreatments which lead to the selective formation of new acidic groups. The precise distribution of these newly-formed acidic groups can be determined by staining adjacent sections not exposed to the pretreatment. The potentialities of this type of procedure are only just beginning to be exploited. The two most useful pretreatments so far studied are the sulphation of polysaccharide hydroxyl groups and the oxidation of protein sulphydryl groups. Both these lead to the formation of highly acidic sulphate or sulphonate groups; so very selective staining can be obtained at a sufficiently acid pH.

The sulphation technique was first investigated in detail by Kramer and Windrum[10] who tried a number of sulphation mixtures and demonstrated the sulphate groups formed by their metachromatic staining with an unbuffered solution of azure A. This original method suffered from several disadvantages which have largely been overcome by Lewis and Grillo[11], who have put forward a method suitable for routine use in which the sulphate groups are demonstrated by staining with methylene blue or another basic dye at a suitably acid pH, and the slides are taken from the anhydrous sulphation medium to water by a method that avoids serious tissue distortion. They propose two sulphation media: (*a*) a mixture of concentrated sulphuric acid and glacial acetic acid to demonstrate all carbohydrate derivatives except glycogen (which is extracted), and (*b*) sulphuryl chloride vapour which gives intense, selective staining of glycogen. These sulphation procedures provide a useful alternative to the periodic acid-Schiff (PAS) method discussed later (p. 60) in that cytological detail is often better and the conditions of sulphation and staining can be varied to suit the particular problem under investigation (*Figure 2.1*).

Several reagents, of which performic acid has proved the most satisfactory, will oxidize sulphydryl or disulphide groups to highly ionized sulphinic or sulphonic acids which can then be demonstrated selectively by staining with basic dyes at a very low pH. Methylene blue at pH 2·5 is adequate for most purposes but even more selective, although fainter, staining can be obtained with alcian blue in 2N sulphuric acid. The alcian blue technique has been used very successfully by Adams and Sloper[12] to investigate the distribution of neurosecretory substance in the hypothalamus. It can also

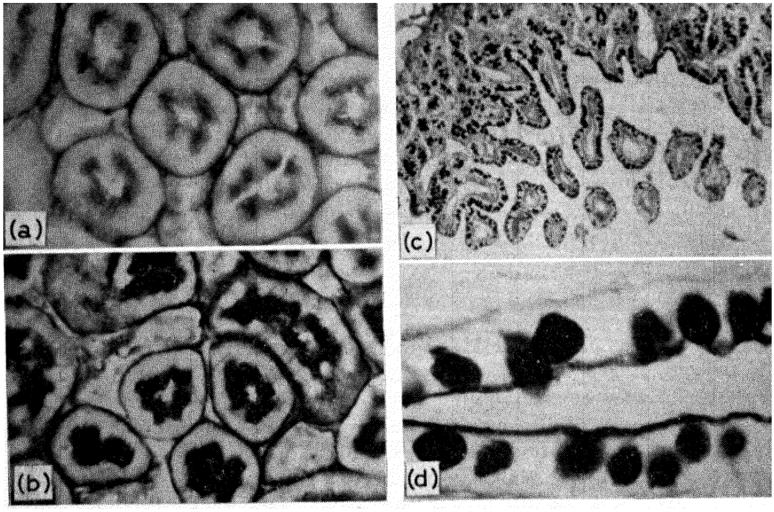


Figure 2.1.[11] Sections of rabbit kidney stained by (*a*) the PAS procedure, (*b*) methylene blue at a pH of 2·5 after sulphation in a 1:1 mixture of acetic and sulphuric acids. Note the sharper delineation of basement membranes and the greater detail in the brush border of the tubular lumen given by the sulphation technique.
Sections of rabbit intestine stained after sulphation in a weaker, 3:1 mixture of acetic and sulphuric acids to show selective staining of mucous cells and their secretion, (*c*) low-power view, (*d*) high-power view of part of crypt.

be used to stain the cystine-rich basiphil cells of the anterior pituitary and any other structures particularly rich in the sulphur-containing amino acids.

A most interesting extension of this technique has been developed by Schriebler and Schiessler[13]. They found that following oxidation by performic acid or acidified permanganate insulin stains metachromatically with pseudoisocyanines. These are basic dyes in which the single net positive charge is shared between two nitrogen atoms some 4 to 5 Å apart, and it is suggested that a substance with a pair of sulphonic acid groups about this critical distance apart is able to hold two dye molecules in the correct position to give the metachromatic colour. This technique is highly selective for insulin among substances likely to be present in biological material. Since there are related dyes with different interchange distances it is very possible that this method can be adapted for the equally specific staining of other cystine-containing substances.

METHODS INVOLVING THE DEMONSTRATION OF ALDEHYDE GROUPS

Of the histochemical reactions in which the chromophore group is bound by covalent links, the one most important and widely applied is that based on the Schiff test for aldehydes. It forms the basis of two very important histochemical techniques: the Feulgen test for deoxyribonucleic acid (DNA) and the periodic acid–Schiff test for carbohydrates, in each of which aldehyde groups are produced from these particular tissue constituents by suitable, selective pretreatment, mild acid hydrolysis in the case of the nucleic acid and periodic acid oxidation in the case of the carbohydrates.

Schiff's reagent is prepared by treating a solution of the dye basic fuchsin with sulphurous acid. Tissue sections are immersed in the resulting colourless solution, well rinsed in sulphurous acid and then washed in running tap-water. Tissue components which possessed aldehyde groups are thereby stained a red or magenta colour which is similar to but not identical with that of the original dye.

In view of the importance of this test in histochemistry it is amazing that we still do not fully understand the detailed chemistry of the reactions involved. The paper usually quoted in the histochemical literature is that published in 1921 by Wieland and Scheuing[14], but some of their conclusions hardly seem consistent with modern theories of organic chemistry. What does seem certain is that when *p*-rosaniline, the principal constituent of commercial basic fuchsin, reacts with sulphurous acid, a sulphonic acid group becomes attached to the central carbon atom. The possibility of resonance about this carbon atom is thereby lost; so the resulting molecule is colourless and no longer carries a net positive charge. Further addition of sulphur dioxide, which exists free in the solution, probably occurs at one or more of the *p*-amino groups to give *N*-sulphinic groups. The resulting molecule couples much more readily with aldehydes than does the original basic fuchsin, but the mechanism of this coupling and the structure of the final addition product are not definitely known. Wieland and Scheuing suggested that the final product contained $-NH-SO_2-CH(OH)-R$ groups in the para-positions. Rumpf[15], on the other hand, was able to isolate intermediate compounds of the type $-NH-CH(SO_2{\cdot}OH)-R$, and it seems more than likely that subsequent loss of sulphur dioxide and water occurs to give the grouping $-N{=}CH-R$. A direct carbon-nitrogen link would seem most likely in view of the high stability of the final product. After condensation with one or more aldehyde groups has occurred, there is a tendency for the sulphurous acid to be lost from the central carbon atom and it is lost completely if the concentration of free sulphur dioxide is decreased, as when the slides are washed in running water. Resonance is thereby restored and colour regained. Our ignorance of some of the detailed chemistry does not detract from the histochemical value of this technique it can be used with confidence to visualize the distribution of aldehyde groups in tissue sections.

Schiff's reagent can be obtained commercially ready for use (under the name Feulgen Reagent) or it can be prepared in a variety of ways[3], all of which depend upon reaction of basic fuchsin with sulphur dioxide in acid solution. The reagent should always be stored at about 4°C in a tightly

stoppered bottle but should be allowed to warm up to room temperature before use: its 'shelf life' varies from a few weeks to many months depending upon the method of preparation. Treatment of sections for 20 to 30 minutes at room temperature appears to give maximum colour development. Removal of excess reagent by careful rinsing in sulphurous acid solution is essential. If any appreciable amount of sulphur dioxide is lost, free *p*-rosaniline is regenerated; this will act as an ordinary basic dye, staining any strongly basiphilic structures a magenta colour, but this is a hazard which is easily avoided by careful technique. The final colour is quite stable to all ordinary counter-staining and mounting procedures.

Other dyes besides *p*-rosaniline can be used to prepare Schiff-type reagents which function in a similar way. Kasten[16] has made an exhaustive survey of over two hundred different dyes. He found that only those with a free amino group can form Schiff-type reagents and he lists a number which are suitable for cytological purposes. Many of them are not completely decolourized by sulphur dioxide so that they also tend to stain basiphilic structures, although only the staining of cartilage is appreciable at the acidity used. It is, therefore, essential to do careful controls with these reagents. Their great advantage is that they widen the range of colours available, the blue ones being especially valuable since they give better visualization of fine cytological detail. I have had most success with a reagent prepared from thionine: the results obtained are sometimes extremely good but are not always reproducible and some batches of the dye do not work at all.

A method which depends upon rather different chemical principles is that introduced independently by Camber[17] and by Ashbel and Seligman[18] in 1949. It warrants a mention here since it illustrates a commonly used method of obtaining chromophore groups which are covalently linked to specific tissue components. The aldehyde groups are condensed with the hydrazide of 2-hydroxy-3-naphthoic acid and the product subsequently coupled with a diazonium salt to give a brilliantly-coloured azo dye. The two steps in the procedure cannot be combined since they require different conditions. The initial condensation occurs best under mildly acid conditions whereas diazonium salts couple best with naphthols at a neutral or slightly alkaline pH. The procedure is therefore more complicated than that with Schiff's reagent. Furthermore, its absolute sensitivity appears to be less and it usually gives appreciable background staining. The general principle of producing an azo dye covalently attached to tissue components possessing some specific group is, however, a very valuable one.

The Feulgen Reaction for DNA

The Feulgen reaction was the first histochemical procedure to make use of Schiff's reagent to demonstrate tissue aldehyde groups and its introduction by Feulgen and Rossenbeck in 1924 can fairly be regarded as marking the rebirth of histochemistry in this century[19]. The reaction depends upon the formation of potential aldehyde groups from DNA by mild acid hydrolysis. For many years the precise mechanism of the reaction and the validity of the results obtained were hotly debated in the literature[3], although there is

now little doubt about either. A DNA molecule is made up of nucleotide units, each nucleotide consisting of a purine or pyrimidine base attached to the 1-position of a 2-deoxyribose group phosphorylated at the 5-position. The individual nucleotides are formed into chains, each phosphate group being linked to the 3-position of the sugar in the next nucleotide. In intact DNA these chains may contain a thousand or more nucleotides. Under mild acid conditions the purine bases are progressively hydrolysed off to reveal the potential aldehyde groups of the first carbon atom of the deoxyribose. With prolonged acid treatment depolymerization occurs, due presumably to the hydrolysis of sugar–phosphate links. Thus there is an optimum hydrolysis time: if the hydrolysis is too short not all the available aldehyde groups are revealed; if it is too long some of the hydrolysed material may be lost from the sections. The optimum time varies with the method of fixation used, for reasons which are not at all clear. Bauer[20] gives a list of suitable times for many of the common fixatives, but when one uses a new tissue for the first time it is as well to determine the optimum value by trial and error. Several alternative methods of hydrolysing off the purine bases from DNA have been suggested, but none of them appear to offer any real advantage over that originally suggested by Feulgen and Rossenbeck.

The specificity of the Feulgen reaction for DNA is extremely high, no other substance yet found in biological material giving free aldehyde groups on mild acid hydrolysis. However, there are three groups of substances which may give a false positive result if adequate controls are not used. In order of their importance as interfering substances, these are: auto-oxidized unsaturated lipids, acetal phospholipids, and a substance at present unidentified found in certain tissue elements notably in rat arterial walls. The first two are normally not encountered in paraffin sections since the offending substances are largely extracted during processing of the material, and the third has a distribution which makes confusion with DNA most unlikely. The presence of any such interfering substance can be detected by staining a slide in Schiff's reagent without prior treatment with hydrochloric acid. If they are present, any risk of confusion can be avoided by blocking the aldehyde groups before carrying out the Feulgen hydrolysis. This is an example of a very general method of proving or of improving the specificity of any given histochemical test. This method has been used to show that in the Feulgen test the organic grouping responsible for the staining with Schiff's reagent is in fact aldehydic, or at least has all the chemical reactions attributable to an aldehyde. Thus a whole range of chemical treatments which should eliminate the characteristic reactivity of aldehyde groups do in fact prevent staining. These same treatments, if interposed before the hydrolysis stage, should block any staining due to preformed aldehyde groups. Pearse[3] lists a total of eleven such treatments and discusses many of them in some detail. However, he makes only brief mention of the one which has proved in practice to be far the most satisfactory. This is the technique, recently introduced by Lillie and Glenner[21], of treating sections with a molar solution of aniline in glacial acetic acid at room temperature. The potential aldehyde groups released from DNA are blocked by this reagent after only 5 minutes treatment and 30 minutes is sufficient for all types of aldehyde

groups except those produced by the action of periodic acid on a few mucins of which that present in gut is an example. For routine purposes 20 to 30 minutes treatment is adequate.

The Periodic Acid-Schiff (PAS) Test

The second important method depending upon the formation of aldehyde groups is the periodic acid–Schiff technique. This method depends upon the fact that aqueous periodic acid selectively oxidizes 1:2-glycol groups to dialdehydes which do not undergo further oxidation:

$$\begin{array}{l} \text{—CH—OH} \\ \quad | \\ \text{—CH—OH} \end{array} \longrightarrow \begin{array}{l} \text{—CH=O} \\ \\ \text{—CH=O} \end{array}$$

Related groupings in which one of the hydroxyl groups is replaced by a ketone, a primary amine or a secondary amine group are also oxidized, but in all cases the hydroxyl group must be free and unsubstituted. Thus the tissue components which would be expected to react are.

1. Polysaccharides
2. Mucopolysaccharides
3. Glyco- and mucoproteins
4. Glycolipids

Nucleic acids do not react because they do not possess pairs of adjacent, unsubstituted, hydroxyl groups, and fully sulphated acid mucopolysaccharides do not react, probably for the same reason. In dilute aqueous solution at room temperature there are very few other groupings which are oxidized by periodic acid, none of which lead to the production of aldehyde groups except the —CH=CH— group of unsaturated lipids and this reacts too slowly to cause serious interference. Thus the PAS technique is a highly specific method of revealing carbohydrate moieties.

Several procedures have been published. The simplest is probably also the best: sections are oxidized for 5 minutes in a 0·5 per cent aqueous solution of periodic acid, well washed in water and then treated with Schiff's reagent or equivalent. Since this procedure has little effect on non-carbohydrates it can be followed by many other staining techniques, *e.g.* those which use basic dyes. Particularly useful combinations for routine histology are Lillie's allochrome method[22] for connective tissue, in which the PAS procedure is followed by treatment with Weigert's iron haematoxylin and 0·04 per cent methyl blue in saturated aqueous picric acid and Pearse's tripas stain in which celestine blue, Meyer's haemalum and orange G are used. For the study of mucopolysaccharide derivatives a very useful sequence is to follow the PAS procedure by controlled staining with methylene blue at one or more pH values in the range 3·7 to 4·5 (*Plate 2.1*(*a*) (*d*)). If the thionine–sulphur dioxide reagent is used in the Feulgen technique this can be followed by the PAS procedure with the orthodox Schiff's reagent.

Since the PAS technique was first introduced in 1946 the general principle has been extended by the use of other oxidizing agents, notably lead tetraacetate and sodium bismuthate. These are said to have certain advantages over periodic acid, although the latter is unlikely to be superseded for routine

use in the general method for carbohydrates. As research tools in the study of specific compounds, however, some of these more selective oxidizing agents are certain to become important in the future.

HISTOCHEMISTRY OF THE NUCLEIC ACIDS

A very wide range of problems has been tackled by the histochemical techniques so far discussed. In this short review it is only possible to discuss a few—preference being given to those which in the author's opinion are of special interest, either because they concern problems of fundamental biological interest or because they illustrate common advantages or pitfalls of such techniques.

The study of the nucleic acids is an excellent example since it is a subject to which histochemistry has contributed much and one which did much to stimulate an interest in histochemistry during the early stages of its modern development. The chemical study of nucleic acids can be said to have begun with the work of Friedrick Miescher in the latter half of the last century. He separated nuclei from pus cells and from these isolated a phosphorous-containing substance he called nuclein. He next showed that a similar substance was present in salmon spermatozoa which contained both an acidic substance and a base which he called protamine. The next important advance was the publication by Altmann in 1899 of a method of preparing protein-free nucleic acids from animal tissues and from yeast. It was soon realized that the nucleic acids from various sources were fundamentally similar although of two clearly distinguishable types. However, the significance of these two types was not realized for many years. Interest during the early part of this century was concentrated on elucidating the chemistry of the nucleic acids isolated from two particular sources, thymus gland and yeast. During this period a quite erroneous idea of the relative distribution of the two types of nucleic acids grew up: the thymus type of nucleic acid was thought to be characteristic of animal tissues and the yeast type of plant tissue. This view was generally held even as late as 1921 by research workers in the field and was preserved in some biochemical text books for more than another decade. Thus we had the odd situation that for some 30 years the biochemistry of nucleic acids was being extensively studied without any real appreciation of their true distribution in cells and tissues. We now know that the distinction between the two types lies in their intracellular distribution--thymus nucleic acid (DNA) being nuclear only, and yeast nucleic acid (RNA) mainly cytoplasmic. Although biochemists began to become suspicious of the old 'animal-plant idea' soon after 1920, its overthrow and replacement by the modern 'nuclear–cytoplasmic idea' was largely the result of histochemical studies. Even today histochemical techniques still provide the main method of studying the cytological distribution of nucleic acids, although cell fractionation techniques have provided some valuable information in recent years.

There are three main histochemical methods available: the Feulgen reaction for DNA; absorption of ultra-violet light; and staining with basic dyes. Since physical techniques are largely excluded from this review as they require knowledge and equipment not possessed by many biologists, this

discussion will be restricted to the purely chemical methods. First in time and importance is the Feulgen reaction, the chemistry of which has already been discussed. Since only DNA reacts positively this technique provides a ready method of studying the differential distribution of the two types of nucleic acid. Furthermore, if the conditions of the reaction are carefully controlled the intensity of colour is proportional to the amount of nucleic acid, and hence makes possible the quantitative estimation of the nucleic acid content of individual nuclei. Microspectrophotometric studies of this type have shown that in a given species the DNA content of individual nuclei is nearly constant in most, possibly all, somatic tissues, the only exceptions being spermatozoa heads which contain only half, and polyploid nuclei, such as occur in rat liver, which contain two or more times the normal amount. This is an observation which has lent very strong support to the idea that DNA is the actual genetic material. It also provides a firm experimental basis for the very valuable method of expressing the concentrations of cell constituents in terms of DNA. The Feulgen reaction also provides a useful method of producing selective staining of cell nuclei in such a way as not to obscure any faint cytoplasmic staining produced by other histochemical methods.

The other chemical method, staining with basic dyes, demonstrates both types of nucleic acid, but is much less specific than the Feulgen reaction. Its specificity can, however, be increased by the use of the enzyme ribonuclease which selectively removes RNA, and its selectivity improved by the use of basic dyes with a special affinity for nucleic acids. Both these are combined in the method used with such success by Brachet: one of a pair of adjacent sections is pretreated with ribonuclease and both are stained in a mixture of methyl green and pyronine. Although the exact chemical basis is still in doubt this mixture, in practice, gives very clear differential staining of the two types of nucleic acid, the DNA in green and the RNA in red. DNA does, in fact, stain with pyronine alone but from the mixed staining solution it preferentially takes up the methyl green. Another selective method is the gallocyanin–chromalum technique which stains both types of nucleic acid blue. It has the virtues of being a progressive stain and of giving very permanent preparations, but for most purposes the methyl green–pyronine technique is still preferred.

Cytochemical studies by Caspersson using ultra-violet microspectroscopy and by Brachet using the methyl green pyronine technique suggested that RNA was somehow intimately connected with protein synthesis. The circumstantial evidence obtained by them was very strong and it has since been amply confirmed[23] by later histochemical work, much of it with Brachet's method, and by recent biochemical studies. Most rapidly growing cells tend to be rich in RNA. This is a generalization which appears to be valid throughout the animal and vegetable kingdoms. Thus it is true of such diverse examples as onion root tips, mammalian embryos and actively multiplying yeasts. It might be argued that these have other things in common beside the need for rapid protein synthesis and the evidence from non-proliferating cells is perhaps more convincing. Apparently without exception, animal cells which are known to be engaged in active protein synthesis contain large amounts of RNA, both in their cytoplasm and in

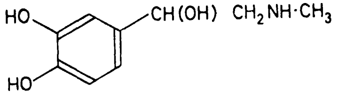
their nucleoli, whereas cells which are highly active in other directions contain much smaller amounts. Thus the peptic cells of the gastric mucosa are intensely basiphilic whereas the oxyntic cells are not. Liver contains far more RNA than kidney. Exocrine pancreas and the silk glands of insects are organs particularly rich in RNA. When endocrine glands are stimulated to secrete, their basiphilia increases if the hormone production involves protein synthesis, as in the thyroid and anterior pituitary, but shows no obvious change in such glands as the adrenal cortex—and there are many other examples where RNA content and the rate of protein synthesis go hand in hand over a wide range of experimental conditions. The degree of correlation found is, in fact, so high that the existence of marked cytoplasmic basiphilia due to RNA in any cell is now taken as strong presumptive evidence that active protein synthesis is taking place there.

Recent biochemical studies have provided quantitative confirmation of many of these histochemical observations, and have also shown that in embryonic development, red cell formation and bacterial growth, an increase in RNA content immediately precedes a speeding up of protein synthesis. However, the most convincing evidence has come from kinetic experiments with purified microsomal fractions prepared by homogenization and ultracentrifugation. Microsomes, which are known to contain much, though not all, of the cytoplasmic RNA, have been shown to be capable of rapid protein synthesis in the presence of suitable precursors, the protein being characteristic of the cell type from which the microsomes were obtained. Brief treatment with ribonuclease is sufficient to destroy this property of the microsomes. It is now seriously considered that the microsomal RNA acts as a template for the highly individual amino acid sequences characteristic of specific proteins. Thus although the role of RNA in protein synthesis has now become a plaything of the biochemist, it was a toy manufactured largely by the histochemist. It is moreover an educational toy of which the histochemist can be justly proud.

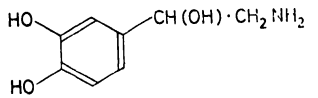
HISTOCHEMISTRY OF PHENOLIC AMINES

Most of the techniques so far discussed are in the nature of general histochemical techniques, applicable to substances present in the majority of cells. There are, however, many tests for substances which are to be found only in a small number of cell types. One such group of substances which has been the subject of much recent histochemical research consists of the phenolic amines derived from the aromatic amino acids tyrosine and tryptophan. Included in this group are several biologically important substances, *e.g.* adrenaline (*I*), noradrenaline (*II*) and 5-hydroxytryptamine (*III*). Much of our knowledge of the distribution of cells containing these substances has been derived from histochemical studies. Attention has been concentrated on two cell types: the adrenal medullary cell which contains adrenaline and/or noradrenaline, and the scattered cells to be found in the intestinal mucosa, known as argentaffin or enterochromaffin cells, which are thought to contain 5-hydroxytryptamine. The histochemical reactions of these substances resemble each other quite closely, and it is

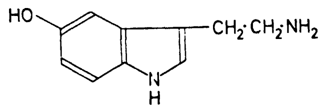
convenient to discuss first of all those tests which are positive with all three substances and then those which give a positive result with noradrenaline or 5-hydroxytryptamine only.



(I) Adrenaline



(II) Noradrenaline



(III) 5-Hydroxytryptamine

Experimental Techniques

The chromaffin reaction

In 1865 Henle observed the formation of a brown pigment in adrenal medullary cells following treatment with dichromate, and early in this century it was suggested that adrenaline was the substance responsible. Later it was shown that other substances reacted similarly provided that they possessed two aromatic hydroxyl or amino groups in ortho- or para-positions to each other, although the precise mechanism and composition of the final product are still unknown. Hillarp and Hokfelt[24] consider that a mixture of 10 volumes of 5 per cent potassium dichromate and 1 volume of 5 per cent potassium chromate, having a pH of about 5·6, is the best fixative for demonstrating the chromaffin reaction in adrenal medullary cells. For routine purposes, however, the halved adrenals should be transferred from it after about an hour to a freshly prepared mixture of 10 volumes of 5 per cent potassium dichromate, 1 volume of 5 per cent potassium chromate, 2 volumes of AR formalin and 7 volumes of water for not more than a day. It is essential that the cells should come into contact with the dichromate before the formalin, but the presence of the latter gives better cytological preservation. The pigment formed by this procedure is easily visible in adrenal medullary cells even in quite thin sections, particularly if they are viewed in blue light (*Figure 2.2*). The pigment appears to be acidic in nature and can be stained with basic dyes, but this does little to increase the over-all specificity of the reaction.

Tests for reducing power

All three substances, like most phenolic amines, are strong reducing agents, and after appropriate fixation (see p. 67) much of this reducing power is retained in paraffin sections. Reduction of silver salts was, in fact, the original diagnostic test for enterochromaffin cells, which for this reason were originally known as argentaffin cells.

There are various silver techniques available and in discussing their relative merits it is necessary to say something about the underlying chemical basis for them. The Masson–Fontana method, in which sections are treated with ammoniacal silver nitrate for 24 hours, is a true argentaffin technique —that is to say, all the metallic silver deposited is formed by endogenous reducing substances present in the section. The Bodian method, developed originally for staining nerve fibres, is really an argyrophil method—after

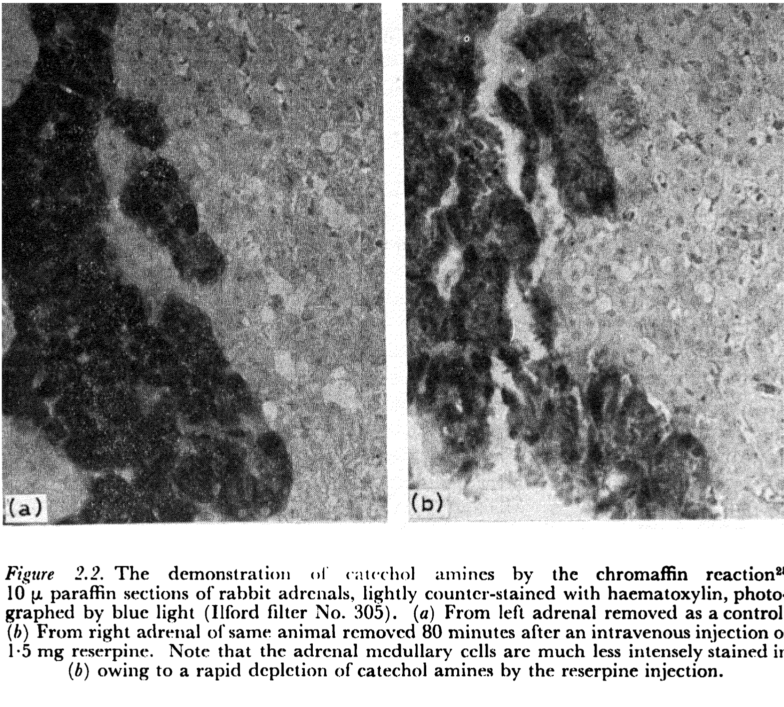


Figure 2.2. The demonstration of catechol amines by the chromaffin reaction[25] 10 μ paraffin sections of rabbit adrenals, lightly counter-stained with haematoxylin, photographed by blue light (Ilford filter No. 305). (*a*) From left adrenal removed as a control. (*b*) From right adrenal of same animal removed 80 minutes after an intravenous injection of 1·5 mg reserpine. Note that the adrenal medullary cells are much less intensely stained in (*b*) owing to a rapid depletion of catechol amines by the reserpine injection.

impregnation in a silver solution the sections are treated with an exogenous reducing agent which causes further deposition of metallic silver, presumably on silver nuclei already formed during the impregnation stage. The Gomori hexamine–silver method is intermediate between these two extremes. Hexamine can act as a mild reducing agent: at a temperature of 60°C marked secondary reduction of silver occurs in a matter of an hour or so; at room temperature secondary reduction is very much less obvious, although it still occurs. In the intestine the results obtained by these various techniques show marked differences. With the Masson–Fontana method only a few scattered cells are shown up and these are the so-called true argentaffin cells. With the Bodian and similar methods very many more cells, often in small clusters, are intensely stained, and these include among them the so-called argyrophil cells. For the strict histochemical demonstration of phenolic amines, and therefore of true argentaffin cells, the Masson–Fontana method is the best. For all other purposes the Gomori hexamine–silver method is

by far the most suitable because of its reproducibility and versatility but the optimum conditions must be discovered by trial and error since they depend upon subjective assessment of the result required. The procedure recommended by Lillie[22] is the best for most purposes though it can be varied to suit individual needs.

Phenolic amines will also reduce ferricyanide ions and this fact provides the basis for a very simple and satisfactory histochemical test (*Plate 2.1(e)*) in which the very insoluble pigment, Prussian blue, (ferric ferrocyanide) is formed in the presence of ferric ions. Various other substances also give a positive result (*e.g.* those containing free sulphydryl groups) but can usually be excluded by other suitable histochemical tests. As with the silver techniques, however, this method is essentially a test for reducing power, a property not exclusive to phenolic amines.

Coupling with diazonium salts

A much more specific technique is based upon the fact that phenols readily couple with diazonium salts to give brightly coloured azo dyes. The precise colour varies with the particular phenol concerned and can be of some diagnostic value in certain instances. The colours given by adrenaline and noradrenaline are indistinguishable from each other, but are very obviously different from that given by 5-hydroxytryptamine. A wide range of suitable diazonium salts is now available commercially. Deparaffinized sections are treated for 1 to 2 minutes with a freshly prepared and filtered 0·1 per cent solution of a diazonium salt in 0·1 N sodium diethylbarbiturate, and washed in running water. With the diazonium salt of 5-chloro-*o*-toluidine (known commercially as Red TR salt) adrenal medullary cells give a rather pale pinkish-orange, but enterochromaffin cells stain a fiery orange (*Plate 2.1(f)*).

Methods specific for noradrenaline

Two histochemical methods of distinguishing between adrenaline and noradrenaline have been developed. The first of these[26] depends upon the fact that noradrenaline reacts with formalin to give an insoluble fluorescent compound, whereas adrenaline does not. Fresh frozen sections are treated with a formol–calcium fixative for a few hours and mounted in glycerol. Cells containing noradrenaline exhibit a strong yellowish-green fluorescence.

The second method depends upon the formation of a brown pigment with aqueous potassium iodate. The best procedure is probably that used by Cattaneo[27]: thin slices of adrenal gland are immersed in saturated potassium iodate (approximately 10 per cent) at a pH adjusted to 9·9–10·4 for 24 hours, and then transferred to 10 per cent formalin for another 24 hours; the tissue is then dehydrated in alcohol, cleared and embedded in paraffin as rapidly as possible. Cells containing noradrenaline are clearly shown up even in quite thin sections. At the alkaline pH used adrenaline does not react.

The Gibbs method for enterochromaffin cells

The best method of distinguishing 5-hydroxytryptamine from the catechol amines is that based upon its reaction with Gibbs' reagent (2:6-dichloroquinone-chloroimide) to form a black indophenol dye. Deparaffinized

sections are immersed for 10 to 15 minutes in a filtered 0·1 per cent solution of Gibbs' reagent in 0·1 N sodium diethylbarbiturate and washed well in running water. With this technique cells containing 5-hydroxytryptamine are stained black whereas those containing adrenaline or noradrenaline are unstained.

Fixation

Fixation is of prime importance in the histochemical study of these substances. Many fixatives either do not preserve them or else destroy their characteristic staining reactions. For the demonstration of adrenaline only dichromate fixation is satisfactory, but because this gives such poor cytological preservation it is usual to include some formalin after the dichromate has had sufficient time to react with the adrenaline. Dichromate also fixes the enterochromaffin substance to give a brown pigment (chromaffin reaction) which has, however, few, if any, of the other staining reactions discussed above. These reactions are retained only if formalin at a not too acid pH is used. After dichromate fixation noradrenaline gives all the reactions shown by adrenaline. Some of this reactivity is also retained after formalin fixation, in particular the ability to reduce ferricyanide and Gomori's hexamine–silver reagent. Thus the effect of fixation upon the staining reactions can sometimes be useful in identifying the phenolic amine which is present.

Identity of the Enterochromaffin Substance

At the beginning of this discussion of the histochemical techniques available for the study of phenolic amines it was implied that the substance present in the enterochromaffin cells was known to be 5-hydroxytryptamine. In fact, this identification rests largely upon the evidence from such tests, and it can be regarded as the major triumph of histochemistry in this field.

The existence of argentaffin cells in the mammalian intestine has been known since the early part of last century. Interest in them intensified with the growth of histochemistry, and there was much discussion of the type of substance which could account for the observed staining reactions. It was soon realized that the substance must be a phenol in nature, and probably had two functional groups, either both phenolic hydroxyls or one hydroxyl and one amino group. Thus Cordier and Lison[28] considered that it was probably a catechol *i.e.* an *o*-diphenol substituted in the para-position and thus structurally very closely related to the adrenergic amines. Gomori[29], however, rejected their conclusion. He considered that the reducing power of the enterochromaffin granules was too weak to be due to an *o*-diphenol. He was the first to use the Gibbs reaction in the study of these cells and he claimed, on the basis of this test, that the para-position must be unsubstituted. He accordingly concluded that the substance was a resorcinol derivative *i.e.* a *m*-diphenol, although in retrospect his evidence for this conclusion was not very strong. Before this paper of Gomori's was published in 1948 two other suggestions had been put forward. Largely on the basis of fluorescence studies Jacobson[30] had suggested a pteridine derivative, a suggestion which has received little support from other workers, and is at variance with much of the histochemical evidence. Erspamer[31], on the other hand, suggested that it was an indoleamine derivative, a suggestion which received little

attention at the time but which has since proved to be correct. It is, in fact, to Erspamer[32] and his collaborators that we owe the eventual identification of this substance. They had earlier made a wide survey of the occurrence of enterochromaffin cells in the animal kingdom and had chosen a few tissues as being particularly suitable sources for the preparation of highly active extracts—foremost among these tissues being the posterior salivary gland of *Octopus vulgaris*. In the final stages of their research they employed paper chromatography and used many of the histochemical tests described above to develop their chromatograms, just as in a tissue section. (This technique could be used much more widely to confirm the identification of substances responsible for histochemical staining.) By this technique they were able to identify enterochromaffin substance as 5-hydroxytryptamine. Almost simultaneously with their work serotonin (a local vasoconstrictor substance isolated from serum) was also identified as 5-hydroxytryptamine. The only reaction given by Erspamer's extracts and 5-hydroxytryptamine that was not given by enterochromaffin cells was the indole reaction—a discrepancy which appears from present evidence to be almost certainly due to the effect of formalin fixation since pure 5-hydroxytryptamine tested *in vitro* will not give the indole reaction after treatment with formalin.

More recent work has made the identification even stronger. Subjects with tumours of the enterochromaffin cells excrete excessive amounts of 5-hydroxyindoleacetic acid which is the normal detoxification product of 5-hydroxytryptamine. Following injection of reserpine, an alkaloid which causes rapid loss of 5-hydroxytryptamine from the intestine, the typical histochemical staining of enterochromaffin cells is no longer detectable although this returns during the subsequent recovery of the tissue level of 5-hydroxytryptamine.

Finally, Benditt and Wong[33] compared the staining of enterochromaffin cells with that of model slides of gelatin solutions containing various concentrations of 5-hydroxytryptamine. Their results strongly favoured Erspamer's identification of 5-hydroxytryptamine as the substance responsible for the histochemical staining. From the intensity of their staining reactions they estimated that the cytoplasmic concentration of 5-hydroxytryptamine in these cells was of the order of 10 mg/ml. This figure was not inconsistent with their estimates based upon the pharmacological assay of whole pieces of intestine and estimates of relative cell volume. From their experiments with gelatin models they concluded that the ferric ferrocyanide reduction test was the most sensitive for 5-hydroxytryptamine and would just detect concentrations of the order of 0·5 mg/ml. The silver reduction test and the diazo reaction were not quite so sensitive, being able to detect about 1 mg/ml. These results explain why it is not possible to detect 5-hydroxytryptamine histochemically in certain other tissues which are known to contain it from pharmacological assays. Thus Mast cells contain approximately 0·5 mg/ml., a concentration too low to be conclusively demonstrated by histochemical means, and platelets which may contain up to about 2 mg/ml., are only very faintly stained. 5-Hydroxytryptamine is also present in certain regions of the brain, notably the area postrema and the hypothalamus, but it would be detectable histochemically only if it was concentrated into a very small proportion of the cells.

The mass of evidence in favour of 5-hydroxytryptamine being the enterochromaffin substance would appear overwhelming. Nevertheless, some workers are still not prepared to accept it at face value, mainly because of certain minor inconsistencies in the histochemical reaction of these cells which are not reconcilable with those of 5-hydroxytryptamine in the test tube or in gelatin models. In a recent paper Lillie[34] has collected together these inconsistencies. However, minor inconsistencies of this type are not uncommon in histochemistry and usually arise because the substance being studied is not in a free form in the cell as it is in the *in vitro* experiments.

We do not yet know the precise form in which 5-hydroxytryptamine exists within the enterochromaffin cells though it is unlikely to be free in the general cytoplasm. Under the light microscope, staining always appears to be granular and in the electron microscope, numerous osmiophile membrane-bound granules are visible in the cell cytoplasm. It is therefore very probable that the 5-hydroxytryptamine is present in much the same form as are the catechol amines of the adrenal medullary cell. There the phenolic amines are contained in small granules possessing a complete investing membrane. These granules can be separated from adrenal medullary homogenates by differential centrifugation and have been found to contain an isotonic solution of amine as a salt with ATP. If 5-hydroxytryptamine is retained in a similar manner its concentration in the granules will be far higher than that used in model experiments, which approximates to the mean concentration per unit volume of cytoplasm. This concentrating of 5-hydroxytryptamine into membrane-bound particles could well explain several of the observed inconsistencies. It is also possible that some of the amine is in a chemically combined form; Erspamer found that many of his extracts contained significant amounts of a material which yielded 5-hydroxytryptamine on mild alkaline hydrolysis.

The Distribution of Catechol Amines

Histochemistry played an essential part in the early studies of the adrenal medulla and of so-called chromaffin tissue generally. Now with the recent development of more refined techniques it has again become of importance in this field. A recent histochemical finding of considerable importance concerns the relative distribution of adrenaline and noradrenaline. It has long been known from pharmacological assays that both amines exist in the adrenal gland and it has been suggested that the proportions released may vary with the type of physiological stimulus applied. The final stage in the biosynthesis of adrenaline is known to be the methylation of noradrenaline, and following depletion of the adrenal medulla the noradrenaline content rises rapidly to a maximum, only to decline as the adrenaline content approaches the normal value. The distribution of noradrenaline has been studied by both the histochemical methods selective for this amine (the iodate and fluorescence methods) which have shown that it is not uniformly distributed in the adrenal medulla but is largely confined to isolated groups or islands of cells which are otherwise indistinguishable from those containing mainly adrenaline. The fact that in some species these cells occupy a characteristic position (*e.g.* around the periphery of the medulla in the

hamster) suggests that their high noradrenaline content is not merely a phase in the synthesis of adrenaline, but is a permanent condition caused by the absence of an active methylating mechanism. Differential release of these two amines is therefore theoretically possible.

Modern histochemical techniques have also been used to study the distribution of phenolic amines elsewhere in the body. Thus, on the basis partly of histochemistry and partly of electron microscopy, it has been suggested[35] that the chemoreceptive cells of the carotid body contain a phenolic amine which may be the transmitter substance that initiates the afferent nerve impulses in response to anoxia. The staining reactions are much weaker than in adrenal medullary cells, presumably because the concentration of amine is much lower; so it has not been possible to identify the substance with certainty, but it is probably either noradrenaline or some substance with very similar chemical properties. Scattered cells in various peripheral tissues have also been detected by histochemical methods for phenolic amines. Thus Falck, Hillarp and Torp[36] have detected cells in certain tissues of ruminants, particularly in the connective tissue of their lungs. The distribution of these cells parallels very closely that of dopamine, determined by pharmacological assay, and their histochemical reactions resemble those of the noradrenaline-containing cells of the adrenal medulla. There can be little doubt that these are the cells which contain the dopamine. Scattered chromaffin cells have also been seen in human skin[37] but here the identity of the phenolic amine present is much less certain.

A recent exciting contribution of histochemistry concerns the relationship of chromaffin tissue to the sympathetic nervous system. It has long been known that the amine content of postganglionic nerves is often much lower than that of the tissue they innervate. One explanation is that the nerve terminations contain a much higher concentration than the main length of the axon. But recent work by Burn and Rand and their collaborators[38] suggests an alternative explanation. They were investigating the pharmacologically paradoxical behaviour of certain tissues in which a response akin to sympathetic stimulation could be obtained by the peripheral application of acetylcholine or of nicotine as well as of adrenaline. Piloerection in the cat's tail gives this type of response, and here they found chromaffin cells at the bases of the arrector pili muscles. Chromaffinity of these cells was markedly reduced by reserpine treatment and partially reduced by sympathetic denervation, both conditions which lead to a fall in the noradrenaline content of the tissue. It is tempting to suggest that these chromaffin cells release noradrenaline which acts upon the arrector pili muscles and that the release is brought about by the action of acetylcholine liberated from the endings of the sympathetic nerve fibres. A similar situation appears to exist in the cat's nictitating membrane and many arterial walls. At present, however, the available evidence is no more than suggestive, and much more critical work needs to be done.

ENZYME HISTOCHEMISTRY

This particular branch of histochemistry has advanced enormously in the last decade. Although there were a few earlier techniques developed,

particularly for oxidases, the subject can be said to date from 1939. In that year Gomori[39] in the U.S.A. and Takamatsu[40] in Japan independently described a method for the enzyme alkaline phosphatase that could be applied to tissue sections. Since that time there has been a rapid development of new techniques so that methods are now available for about fifty enzymes, in some cases there being two or three possible methods for the same enzyme. Enzymes which can be studied by existing techniques are largely confined to two main groups, oxidative and hydrolytic. Attention here will be concentrated on the second group, not because the oxidative enzymes are considered to be unimportant, but because the histochemical study of the hydrolytic enzymes is beginning to yield such interesting and far-reaching results.

The first method developed for alkaline phosphatase, now commonly called the Gomori technique, consists in incubating tissue sections in a medium containing sodium β-glycerophosphate as substrate and a high concentration of calcium ions buffered to about pH 9. Phosphate ions liberated by enzymic hydrolysis are 'captured' by the calcium ions, insoluble calcium phosphate being deposited at or near the sites of enzyme activity. In the original technique the invisible deposits of calcium phosphate were revealed by the von Kossa technique in which they were replaced by silver phosphate and black metallic silver produced by the action of sunlight. Gomori later modified this part of the technique and in the modern version[3] the incubated sections are treated first with a solution of a cobaltous salt and then with yellow ammonium sulphide so that the calcium phosphate precipitate is converted into black cobalt sulphide. The general principle of this technique has been utilized for the study of many other enzymes—for other phosphatases with various substrates and either calcium ions at an alkaline pH or plumbous ions at an acid pH to capture the liberated phosphate, and also for carboxylic esterases with cobaltous ions to capture the liberated free fatty acid.

In 1944 another method for alkaline phosphatase depending upon quite different chemical principles was published by Menten, Junge and Green[41]. The original method was most unsatisfactory but within a very few years the general principle had become the most important in enzyme histochemistry[42]. Basically, the technique consists in incubating sections in a medium containing the appropriate ester of α-naphthol (sodium α-naphthyl phosphate for alkaline phosphatase or α-naphthyl acetate for many esterases) and a suitable diazonium salt. At a neutral or alkaline pH the diazonium salt very rapidly couples with any free naphthol liberated to give an insoluble azo dye. Since the azo dye is brightly coloured no further treatment is required—the sections can be watched under the microscope and when adequately stained are simply washed in water. A great many enzymes will hydrolyse the appropriate naphthyl ester almost as rapidly as the normal, physiological substrate, so this basic method has a very wide application.

In 1949 Koelle and Friedenwald[43] introduced a method specific for the cholinesterase group of enzymes. Again the original method left much to be desired but subsequent modification has made it a most valuable tool for the study of this important group of enzymes[44]. In this technique tissue sections are incubated at a somewhat acid pH with acetylthiocholine as

substrate and copper ions as the capturing agent; glycine is usually also added. A copper–glycine complex is formed whereby the medium can contain sufficient copper without the concentration of free copper ions becoming too high. Enzymic hydrolysis liberates free thiocholine which forms a white, insoluble complex with the copper. Subsequent treatment of the sections with a sulphide solution converts this complex into dark brown copper sulphide.

Another, entirely new method for esterases was described independently by Barrnett and Seligman[45] in 1951 and by Holt[46] in 1952. Initial results were very disappointing but the chemical basis of the method has since been studied, by Holt and his collaborators, more thoroughly than that of any other comparable technique, so that it is now capable of very precise localization and offers great promise for the future. The method depends upon the use of indoxyl esters. Enzymic hydrolysis liberates free indoxyl which is a highly labile substance that readily undergoes rapid oxidation to yield the very insoluble and brightly coloured dye indigo. With simple indoxyl esters localization is very poor, the indigo being deposited in the form of large crystals. However, very good localization can now be obtained by the use of suitable conditions for the oxidation and of indoxyl esters substituted in the benzenoid ring (5-bromo-4-chloroindoxyl acetate being the best esterase substrate)[47]. Unfortunately, such compounds are very difficult and expensive to prepare and they have not been widely used so far.

Finally, in 1955, Crevier and Bélanger[48] introduced a method which they hoped was specific for true cholinesterase. It depends upon the fact that this enzyme will hydrolyse thioacetic acid to give acetic acid and hydrogen sulphide, the latter forming highly-insoluble, black lead sulphide in the presence of a low concentration of plumbous ions. Relatively little work has been done with this technique, so its specificity and general validity are still in doubt[49] but it promises to become a most useful method, particularly for electron microscopic studies[50].

Consideration of General Principles

It has already been noted that most of the techniques gave unsatisfactory results when they were first introduced and they had to be extensively modified before becoming suitable for general use. This is indeed typical of enzyme histochemistry as a whole for it has developed in a very empirical fashion. Only in recent years has a serious attempt been made to place the methods used on a firm physical basis[51,52] and unless we understand the physical principles involved we shall never be able to realize the full potentialities of these techniques. Fortunately, although the methods for hydrolytic enzymes involve a wide range of chemical reactions, they depend upon similar chemical and physical principles; so data obtained on one technique can often be applied to the others. For the discussion of these general principles it is convenient to divide the histochemical reactions into two phases:

1. Hydrolysis of the substrate by the enzyme to give the primary reaction product (PRP)
2. Capture of the PRP and precipitation of the resulting final reaction product (FRP)

Hydrolysis of substrate

Where the substrate used is very different from any substance which is likely to be encountered in nature, a histochemical technique may have a specificity which cuts across the enzyme classifications of the biochemist. Thus, of the various methods for the demonstration of esterases only that for thiocholine has a clearly defined specificity. The use of selective inhibitors only partially solves this problem: what are really needed are detailed studies by combined histochemical and biochemical techniques.

A much greater problem is that of obtaining a degree of histochemical staining which is proportional to the amount of enzyme. Unless this can be achieved the final result is not a true representation of enzyme distribution. The conditions which must be fulfilled have been considered by Holt and his collaborators in a number of recent papers[47,51,53]. They have been largely concerned with indoxyl methods but most of the theoretical conclusions can be applied to other enzyme techniques. For the hydrolysis stage, the necessary condition, expressed in physico-chemical terminology, is that zero-order kinetics should be obeyed, that is, the rate of hydrolysis should be independent of the substrate concentration and directly proportional to the enzyme concentration. For an enzyme present in the extracellular space of a tissue this condition should normally hold, but for an intracellular enzyme it may break down at sites of high enzyme activity. Thus, if the cell membrane is sufficiently impermeable the supply of substrate to the interior of the cell may become the limiting factor. To take an extreme example, imagine two cells identical except that one contains ten times more enzyme than the other; if the smaller amount of enzyme is still sufficient to hydrolyse the substrate as rapidly as it diffuses into the cell, the two cells will appear equally stained by the histochemical procedure. Thus it can be seen that inadequate diffusion of substrate will always lead to loss of contrast, sites of high activity being less intensely stained than they ought to be. Most membranes owe their permeability properties to their lipid component; so alcohol or acetone-fixed, paraffin-embedded material is unlikely to contain any significant diffusion barriers. In formalin-fixed, frozen sections, however, there may be appreciable hindrance to diffusion particularly of substances with low lipid solubility. It is difficult to assess the importance of this effect but certainly in many histochemical techniques contrast is greater when enzyme activity is depressed by, for example, partial inhibition or adverse pH. In unfixed tissues the impermeability of intact membranes can be a very serious source of error.

The capture reaction

This is the crucial stage in most histochemical techniques. Ideally, first-order kinetics should be obeyed although small deviations are unlikely to be very serious in practice. This condition will usually hold, but only if the capturing reagent is everywhere present in an adequate concentration; so here again the existence of diffusion barriers may cause serious errors. Even more important is the actual velocity of the capture reaction. The more rapid this is, the shorter the time which the PRP has to diffuse away from

its site of formation, and the more nearly will the spatial distribution of the FRP reflect that of the enzyme. Thus it is this stage that limits the accuracy with which the site of enzyme activity can be localized.

Fortunately, this problem can be analysed mathematically within certain limits. The most detailed paper on the subject is that by Holt and O'Sullivan[53] in which they consider the distribution of the FRP to be expected theoretically in and around a spherical enzyme site set in an infinite medium. Their basic assumptions are that the enzyme is uniformly distributed inside the sphere, with zero concentration outside, and that the inward diffusion

Table 2.1. Variation in percentage of stain deposited in a site of radius 1 μ (After Holt and O'Sullivan[53])

D (cm^2 sec^{-1})	k (sec^{-1}) 10^4	10^3	10^2	10	1	0·1
10^{-5}	57·2	19·0	3·5	1·4	0·1	0·01
10^{-6}	85·1	57·2	19·0	3·5	1·4	0·1
10^{-7}	95·3	85·1	57·2	19·0	3·5	1·4
10^{-8}	98·5	95·3	85·1	57·2	19·0	3·5
10^{-9}	99·5	98·5	95·3	85·1	57·2	19·0
10^{-10}	99·9	99·5	98·5	95·3	85·1	57·2

of substrate is sufficient for its hydrolysis to be everywhere proportional to the concentration of enzyme. They then constructed a table showing the fraction of the FRP which would be deposited within the spherical site for a series of values of k, the first-order velocity constant of the capture reaction, and of D, the diffusion coefficient of the PRP. This table is so important for the understanding of the limitations to be expected in enzyme localization that it is reproduced in full (*Table 2.1*). The velocity constant equals the reciprocal of the time at which only $1/e$ of the PRP remains uncaptured and has the dimensions sec^{-1}. The diffusion coefficient has dimensions cm^2 sec^{-1}. The fraction of stain deposited within the site is a function of k/D; so any particular value is repeated along the descending diagonal from left to right across the table. The actual figures were calculated for a site of 1 μ radius but they can be applied to other-sized spherical sites: since k/D has the dimensions cm^{-2}, all the figures are moved one place to the right or one place upwards in the table for a site of radius $\sqrt{10}$ μ, two places in the same direction for a site of radius 10 μ, two places in the opposite direction for a site of radius 0·1 μ, and so on.

Most of the capture reactions used in histochemistry have velocity constants which are probably not much in excess of 10 sec^{-1} and the PRP is usually a fairly small molecule which should have a diffusion coefficient of the order of 10^{-6}. If these values were correct one would expect the fraction of stain in a 1 μ site to be only 3·5 per cent and in a 10 μ site to be about 57 per cent. In practice, localization is obviously considerably better than these figures would suggest. The capture reaction is unlikely to be more rapid than expected, so one suspects that diffusion of the PRP in tissue sections is considerably slower than in free solution. This is not an unlikely possibility since proteins are known to adsorb many substances, particularly those with polar groups. Thus it is probable that the sharpness of the

localization obtained with many histochemical techniques depends upon the adsorption of the PRP by tissue structures. Experimental work on simultaneous coupling azo dye techniques tends to confirm this: the sharpness of localization of esterases obtained with a series of acetates of substituted naphthols as substrates tends to parallel the substantiveness of the free naphthols for proteins rather than the speed of coupling with diazonium salts; lowering the temperature, which reduces the speed of coupling but increases the degree of adsorption, also improves localization.

Diffusion artefacts may be caused not only by slow capture of the PRP but also by slow precipitation of the FRP. Many factors may influence the speed of this precipitation, not the least important being the tendency for molecules to become adsorbed by surfaces; so again the adsorptive properties of proteins and other tissue components may influence the final result. Although no detailed analysis has yet been made it seems likely that slow precipitation will have much the same effect as slow capture. In techniques where treatment after incubation is necessary in order to visualize the initial precipitate further possibilities of error arise. Certainly, unless care is taken, some diffusion can occur at this stage, but with presently accepted techniques no evidence has been produced for diffusion artefacts appreciable at the light microscope level. As an alternative, the initial precipitate can often be detected by phase contrast microscopy but this method has not found much favour because resolution is poor and photography is difficult.

Thus the calculations of Holt and O'Sullivan do not take into account all the stages at which possibilities of diffusion may affect the final picture. However, diffusion of the PRP is probably the most important factor and even though theory and practice do not agree these calculations are valuable because they give a measure of the extent to which localization may be falsified by selective adsorption. Artefacts due to this cause can unquestionably occur. In both the Gomori technique for alkaline phosphatase and the Koelle technique for cholinesterase, staining of nuclei is often observed in the neighbourhood of sites of high enzyme activity, particularly when the conditions of incubation are not optimal. Nuclear staining in the Gomori technique was the subject of a long and sometimes bitter dispute in the literature. Now there seems to be no doubt that in the vast majority of tissues it is a diffusion artefact. If the pH of incubation is reduced from 9·3 to below 8·5 the staining of nuclei becomes more intense and is obvious at greater distances from the sites of high cytoplasmic staining, indeed an inactivated section laid on top of an active one during incubation will acquire some nuclear staining. Nuclear staining becomes obvious even at pH 9·3 if the calcium concentration is lowered. With the Koelle technique nuclear staining is sometimes seen when thick sections are used and can always be produced by lowering the concentration of cupric ions in the incubation medium, *e.g.* by raising the concentration of glycine which chelates with the copper, or by raising the pH which increases the degree of chelation. Thus conditions which decrease the rate of the capture reaction and/or increase the solubility and diffusibility of the PRP or FRP are precisely those which in practice favour nuclear staining. Furthermore, with both techniques, conditions which produce appreciable nuclear staining

give noticeably less precise localization of sites of high cytoplasmic or extracellular activity. Even though nuclear staining is not detectable under optimum conditions it is still possible that other structures with an especially high adsorbing capacity for the PRP, FRP or the capturing species could be causing some false staining within a few microns of genuine sites of high activity and this possibility should always be borne in mind when interpreting faintly stained structures close to intensely stained ones.

One fact which the calculations of Holt and O'Sullivan make very plain is the extreme care which must be taken in the design and interpretation of histochemical techniques for the demonstration of enzyme distribution at the electron microscopic level. None of the capture reactions so far used appear likely to prevent diffusion over distances considerably less than 1 μ; so selective adsorption is likely to be important in determining the final distribution of stain. This is not to say that the electron microscopic picture of enzyme distribution may not be accurate in particularly favourable circumstances. Thus, where the enzyme is confined in small membrane-bound granules, if the membrane is permeable to substrate and capture reagent but not to PRP or FRP, the final stain will also be restricted to the granules. Holt[54] has recently obtained some very convincing electron micrographs showing acid phosphatase (as revealed by a method in which the liberated phosphate ions are precipitated as lead phosphate) sharply confined to particular small cytoplasmic inclusions, a distribution entirely consistent with biochemical findings.

It should not be assumed from the foregoing discussion, however, that diffusion artefacts are a consistent or even a common feature of histochemical techniques. Thus there are many examples where two or more techniques depending on quite different chemical principles give precisely the same result for the distribution of a particular enzyme, and others where the same basic technique with two slightly different substrates gives totally unrelated distributions for two different enzymes.

Preparation of tissues

The method of preparation will depend to some extent on the tissue and to a very great extent on the particular enzyme being studied. If fresh, unfixed tissue is used full enzyme activity is preserved but the material is difficult to handle and many enzymes will diffuse from their original positions during the histochemical processing. If the tissue is fixed before sectioning most enzymes will be retained in position but some loss of activity is almost inevitable. Most techniques are therefore a compromise between translocation of enzyme and loss of activity, the best compromise depending upon the problem being investigated. For the study of many cytoplasmic enzymes which are present in soluble form (*e.g.* non-specific ali-esterase) fixation is essential. For the study of insoluble enzymes or ones confined in cytoplasmic granules fixation is often unnecessary but special precautions must be taken to preserve tissue integrity (*e.g.* the use of sucrose or polyvinyl pyrrolidone) to 'protect' mitochondria). Wherever possible it is desirable to use fixation at least in the initial stages of an investigation but it must always be remembered that the value of the results is thereby limited. Preferably more than one method of preparing the tissue should be used.

Some enzymes will not withstand any form of fixation and if sections are required these must be cut from fresh-frozen tissue inside some form of cryostat. Only relatively few enzymes, *e.g.* alkaline phosphatase, withstand paraffin embedding, the best fixative being ice-cold absolute acetone or alcohol. For most other enzymes the best type of fixation technique is a few hours at 4°C in 10 per cent formalin (or formol–saline) at a pH in the range 6·5 to 7·5. Various methods have been suggested for improving the sectioning of formalin-fixed material on the freezing microtome without further loss of enzyme activity: tissues can be soaked for a few hours in 20 per cent ethyl alcohol or for 24 hours in 0·88M sucrose containing 9 g/l. of gum acacia, or they can be embedded in an alginate gel. Alternatively the tissue can be embedded in a water-soluble poly(ethylene glycol) wax and sections cut at room temperature on an ordinary, paraffin-type microtome.

For many investigations sectioning is unnecessary. Sometimes much more information can be gained from a few carefully teased preparations than from many series of frozen sections. All the techniques discussed can be applied with only slight modification to very small teased pieces of tissue, although prior fixation is usually necessary in order to destroy the impermeability of intact membranes. The rate of diffusion of solutes, such as the substrate and the capturing reagent, decreases very rapidly as the distance to be diffused through is increased. Nevertheless some of the techniques for hydrolytic enzymes have been successfully adapted for the treatment of small pieces of unteased tissue[44,55]. The great advantage of such bulk-staining methods is that they make it easy to appreciate the three-dimensional distribution of enzyme with a clarity not possible in sectioned material. They also make possible the study of tissues which do not cut well on the freezing microtome (*Figure 2.4*).

Some Results of Biological Interest

There is now a vast literature on enzyme histochemistry, much of it highly specialized but some of it with considerable general interest to the biologist. Widely different types of investigation are represented.

Many papers are concerned with methodology—with the adaptation of existing techniques to detect new enzymes, or with minor modifications of accepted techniques for particular purposes. These need not be discussed in detail here, although some have been, and will be, referred to incidently. Early in the history of enzyme histochemistry many papers were merely general surveys of the distribution in tissues of a particular enzyme, or of the enzymes giving a positive reaction with a particular technique. At that stage in the development of the subject such studies were perhaps necessary, since our knowledge of enzyme distribution and of the potentialities of the new techniques was very scanty. And in any field of endeavour exploration for exploration's sake is a necessary and important activity, but it can all too easily attain exaggerated proportions—it can become a fashion. It is therefore gratifying that the number of such papers has declined in the last two or three years. Attention has, in fact, turned more and more to the use of these histochemical methods as an aid to the study of other types of problems in the fields of cytology, physiology, embryology and so on.

The distribution of alkaline phosphatase

An enzyme whose general distribution has been exhaustively studied is alkaline phosphatase. The significance of many of the findings is still obscure because, apart from its presumed role in calcification, the true function of this enzyme is still unknown. Its distribution discovered histochemically may one day provide the clue to this, and certainly any theory of its function put forward must account for the histochemically ascertained facts. It is difficult to produce any logical classification of the sites where this enzyme is found, but on one simple classification it would appear that it occurs at four types of situation where the following active processes are taking place: calcification, solute absorption, protein synthesis and histogenesis.

The presence of alkaline phosphatase during calcification was first suggested by Robison[56] in 1923. He demonstrated it in bones from severely rachitic rats by a procedure which on a macro-scale was the forerunner of Gomori's histochemical technique. With more modern methods it has been possible to follow the events associated with calcification in much greater detail. It has now been demonstrated many times in the bones and teeth of mammals, birds and fish, that alkaline phosphatase activity rises to a very high level immediately prior to the onset of primary calcification, and that this zone of intense extracellular activity retreats in front of the wave of calcification. This association of enzyme activity with calcification can be particularly clearly shown in sections of embryonic limbs treated first with the azo dye technique for alkaline phosphatase and then the von Kossa technique to reveal calcium deposits (*Plate 2.1*(*g*)). There is still some disagreement over the role of this enzyme but it seems probable that its major function is to release from various esters sufficient free phosphate ions to cause precipitation of calcium phosphate; its extracellular distribution and time of appearance are consistent with this idea.

Alkaline phosphatase is present in high concentration in both kidney and intestine where it is entirely intracellular and is sharply confined to the brush border which faces towards the lumen. As far as I am aware this type of distribution has been found in all species so far examined, and the enzyme appears to be always associated with cilia and brush borders, *e.g.* choroid plexus. Its primary function is likely to be similar in all these situations, and one obvious biological activity common to them all is the transport, particularly the active absorption, of solutes. Many people consider this to be an important clue to its function. If so, alkaline phosphatase must be associated with the absorption of non-electrolytes, such as glucose, rather than of electrolytes, such as sodium or potassium ions. In fact, in the mammalian kidney its distribution is very striking with a high activity in the proximal tubules but with negligible activity in the distal ones, and tissues such as nerve and muscle which are known to be active transporters of ions contain very little of the enzyme.

Alkaline phosphatase is also usually associated with sites where rapid synthesis of protein, particularly of fibrous protein, is occurring. The most striking examples are the silk glands of spiders and caterpillers. It is also present during wound healing and during the laying down of the fibrous matrix of bone which appears well before calcification begins. Cilia and brush borders also appear to have a fibrous protein basis and this association

suggested to Goldacre[57] that active absorption of small molecules might involve contractile fibrous proteins as a fundamental part of the mechanism. The basic idea is that at the surface of absorptive cells there exists a protein which can be in two geometrically different forms with differing affinities for a particular solute, *e.g.* glucose.

If this protein is actively converted from one form to the other at the inside of the cell surface and back again on the outside, a large concentration gradient of the solute could be built up across the cell membrane. It is postulated that alkaline phosphatase is involved in either the folding or the unfolding that converts one form of the protein into the other. Most of the esters acted upon by alkaline phosphatase liberate about 3,000 cal/mole on hydrolysis. If most of this energy is transferred to the secretory or absorptive process it would be quite adequate to account for the observed degree of concentrating power possessed by epithelia with a high enzyme activity. This line of reasoning is not in itself, of course, evidence in favour of the protein mechanism and suggests a possible, more general role for the enzyme. It is now known that adenosine triphosphate (ATP) is the immediate source of energy for the performance of useful work by many cells—to quote but two examples, in muscle contraction and in gastric secretion. In both of these a large proportion of the energy released by the hydrolysis of ATP is utilized in the performance of useful work. When the enzyme responsible for the hydrolysis is detached from the cellular structure and purified, the energy released is no longer used but is wasted as heat. The fact that purified alkaline phosphatase *in vitro* appears to be a simple hydrolysing enzyme is therefore in no way evidence against it having an energy-transferring role in the intact cell. If this was its major role, its intracellular localization in many cells would be explicable. Although the pumping of ions usually appears to require an ATP-splitting enzyme, alkaline phosphatase could provide sufficient energy for the transport of non-electrolytes. Its association with fibrous proteins is not so easy to explain. Recent biochemical evidence makes it almost certain that the energy for the formation of peptide bonds is derived from ATP, but it is quite possible that for the formation of the weaker bonds which cross-link adjacent peptide chains the energy released by the action of alkaline phosphatase would be sufficient. Alkaline phosphatase might be, in fact, the 'poor man's ATPase', to be used for the driving of reactions which require smaller quanta of energy than are released by the hydrolysis of ATP.

A fourth type of location where alkaline phosphatase is seen— the developing embryo at certain stages of histogenesis—has not perhaps received the amount of attention it deserves. There are many hints to this effect scattered through the literature but no attempt appears to have been made to study the specificity of this association in any detail. One particular example, not previously published, occurs in the development of the otic vesicle in the chick (*Figure 2.3*). This vesicle is formed by invagination of the surface epithelium overlying the presumptive otic region. The epithelium has at first no generalized alkaline phosphatase activity, but when the slight concavity becomes apparent the cells in this region acquire significant enzyme activity concentrated towards their outermost margins. Activity rises as invagination continues; its distribution remains similar but more

sharply marked so that the appearance is not unlike that seen in mature proximal renal tubules since the original outer surface of the cells is now facing the developing cavity. By the time invagination is complete, the cells lining the otic vesicle proper have lost most of their enzyme activity, which

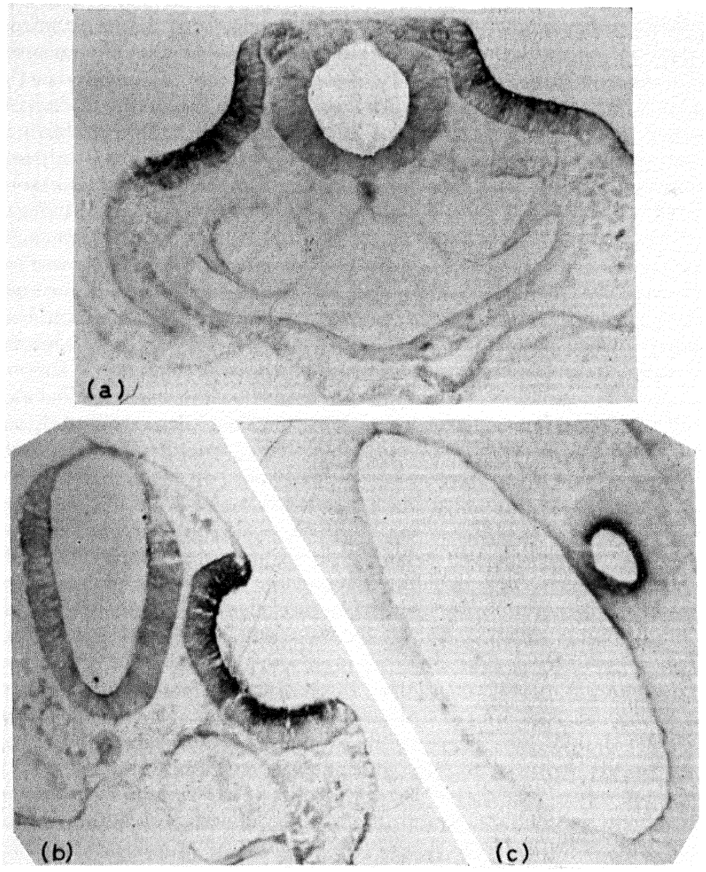


Figure 2.3. The distribution of alkaline phosphatase in the developing otic vesicle of the chick, as shown by the simultaneous coupling azo dye technique applied to paraffin-sectioned material fixed in cold acetone[58]
(*a*) 2-day-old embryo (14-somite stage) showing first signs of a depression forming in the surface epithelium, alkaline phosphatase activity being confined to this region. (*b*) 2-day-old embryo (16-somite stage) showing invagination well under way, with enzyme activity clearly concentrated at the concave border of the epithelium. (*c*) $4\frac{1}{2}$-day-old embryo in which otic vesicle is now closed, with negligible activity in vesicle itself but high activity along the duct.

is retained only by those lining the otic duct. As a general rule alkaline phosphatase appears at the concave surface of epithelia undergoing invagination whether or not it is also present when the organogenesis is complete (as in proximal tubules). Its role in such situations is obscure: it might be

required for secretion or synthesis needing only low quanta of energy and its prevalence in embryos might be a reflection of their largely anaerobic mode of metabolism. (It is just possible that the enzyme participates in the mechanics of cell movement during invagination: if a progressive contraction of cell protoplasm develops somewhere on only one surface of a growing epithelium, a concavity will develop with an ever-increasing curvature as a geometrical necessity.) Whatever the functional reason, this existence of alkaline phosphatase at the concave surface of an invagination could be used to trace backwards through a graded series of embryos the precise origins of structures formed in this way, but no attempt has so far been made to do so.

Studies on cholinesterase

True cholinesterase, or acetylcholinesterase, is another enzyme which has received a great deal of attention from histochemists. Here the situation is quite different: its major function in animal tissues is certainly known—it brings about the rapid inactivation, by hydrolysis, of the acetylcholine released at the terminations of cholinergic neurons. A recent symposium gives a very good account of much of the histochemical work done on this enzyme[44].

Histochemical techniques for acetylcholinesterase have revolutionized the study of the motor innervation to skeletal muscles. The enzyme activity of most motor end-plates is so high that a few minutes' incubation during the azo dye or a quarter of an hour during the thiocholine technique suffices to show them up in sharp contrast, in a way quite impossible by any of the older histological methods. Applied to whole muscles or strips of muscles these techniques have been used to compare the innervation patterns of different muscles in the same species, of related muscles in different species, and of a particular muscle at different stages of development. Several interesting facts have emerged from these investigations. From them it is now clear that the classical picture of each muscle fibre being innervated through a single large motor end-plate situated somewhere along the length of the fibre is only partly true.

In mammals, a proportion of the ordinary twitch fibres have two end-plates, though whether these are innervated by branches of the same motor neuron is still an open question. In birds, this kind of double innervation is rather more common and some avian muscles have a quite different pattern in which individual fibres have many small myoneural junctions scattered along their lengths. Such multiply innervated muscles are believed to be important in the maintenance of postural tone, undergoing slow contractures rather than rapid twitches when they are stimulated. a similar pattern of innervation is seen in mammalian muscle spindles. These findings of double and multiple innervations are in complete accord with recent physiological studies for which they have provided a firm anatomical basis. The new enzyme techniques have been particularly useful in the study of the multiply innervated fibres since the endings are so small and simple in form that they are very difficult to recognize by the older methods. In mammalian (and probably in other) embryos functional myoneural junctions, as judged by the presence of reflex contractions, are established long before the complex

histologically-recognizable structures of the mature motor end-plate have appeared, so here again enzyme techniques are proving invaluable.

In lower vertebrates a number of quite new and unexpected observations have been made. Thus, 'basket endings' at the ends of segmental muscle fibres in several species were described as early as the latter part of last century, but were always assumed to be sensory in function. The newer histochemical techniques showed, however, that in an amphibian, *Xenopus laevis*, such endings were associated with a high concentration of cholinesterase[55] which was present nowhere else along the length of the muscle fibre. Following upon this initial discovery it was soon shown that terminal innervation was quite common among lower vertebrates, and constituted the sole motor supply to the axial musculature of some species. It was also found that during normal development and during regeneration some muscles at first receive only a terminal nerve supply, and only later acquire the classical pattern of innervation with end-plates along the lengths of the muscle fibres (*Figure 2.4*).

Some neurons possess cholinesterase activity not only at their terminations where it is extracellular, but also along their whole length where it is intracellular. The function of the intracellular enzyme is unknown but recently it has been suggested on a number of occasions that the enzyme might be synthesized in the cell body and slowly transported down the axon for eventual release at the terminations. Axonal flow is not a new idea and true cholinesterase is known to be a very stable enzyme. Although the distribution of enzyme in both normal and experimental material is consistent with axonal flow no conclusive proof of the idea has yet been obtained. Nevertheless it continues to be a very fruitful working hypothesis which has already initiated a number of new discoveries in the field of neurology.

Although most neurons contain some cholinesterase, certain ones contain particularly large amounts and these can be selectively stained by the thiocholine technique if the incubation medium is made sufficiently acid. Such neurons are generally assumed to be cholinergic and on the present available evidence this would seem a reasonable assumption to make in any particular case until proved otherwise. Whatever the underlying reason for their high enzyme activity, however, these nerve fibres can be traced more easily by the thiocholine technique than by any other method, and this fact has been utilized in recent years, to study the peripheral innervation of a number of glands and other tissues.

To do this a much lower pH is used for the incubation medium, as low as 5·0 or below for some species. Under these conditions enzyme activity is depressed and, with suitable incubation times, sites of particularly high activity are shown up in sharp contrast. An obvious and early use of this technique was the disentangling of the known cholinergic innervation to such structures as the salivary glands[44] since sensory and other non-cholinergic nerves remained unstained and so did not confuse the issue. The results have to be interpreted with some caution since certain sensory fibres do stain, particularly those to specialized receptors such as Pacinian corpuscles. The technique has also been used to study sympathetic autonomic ganglia. While most of the cells are negative or only weakly positive a small proportion are often intensely stained, the proportion depending markedly upon both

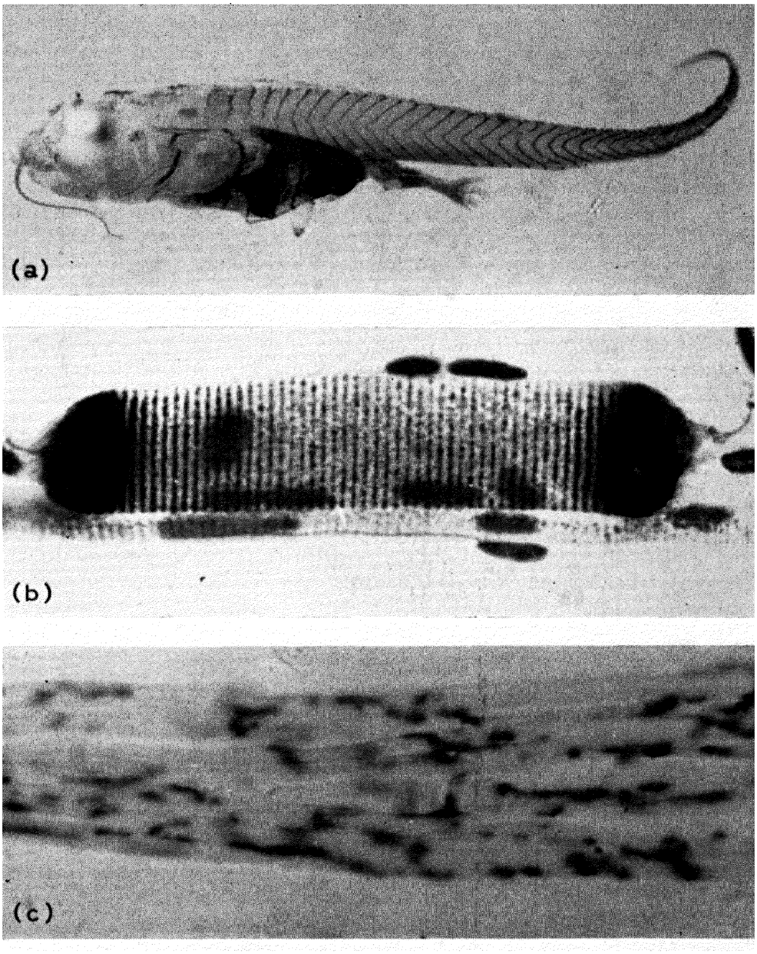


Figure 2.4. Examples of the cholinesterase distribution in the tadpole of *Xenopus laevis* demonstrated by the bulk-staining modification[55] of the simultaneous coupling azo dye technique[59]

(*a*) Whole skinned tadpole, showing myocommatal distribution of enzyme in the axial musculature and septal distribution in the abdominal muscles (the background staining over the abdomen is due to melanin). (*b*) A single muscle fibre teased from the tail and afterwards stained by the Bodian technique for nerve fibres, showing enzyme activity concentrated at the ends of the fibre associated with terminal innervation. (*c*) Part of the lateral segment of the transverse ventralis muscle, showing the profuse pattern of motor-endings.

the ganglion and the species. The significance of these intensely-stained cells is not yet clear: the histochemical data alone are inadequate proof that the cells are cholinergic although there is a strong possibility that they may be. The existence of cholinergic postganglionic sympathetic fibres would be consistent with the suggestion of Burn and his collaborators[38] discussed in the section on phenolic amines (see p. 70). A small proportion of dorsal root fibres are also positive and these may well be cholinergic since it has long been known that dorsal roots possess appreciable choline acetylase activity, the enzyme responsible for acetylcholine synthesis.

This technique has been in use for only about three or four years and more data are required but its use seems likely to add considerably to our knowledge of the peripheral nervous system. Its use in the central nervous system is more recent still but the results promise to be even more important.

It was reasoned[60] that since the preganglionic parasympathetic fibres to the salivary glands in the rat are intensely stained along their peripheral course it should be possible to follow them back into the central nervous system by the same technique. It was found that at a suitably acid pH (about 4·8) the salivatory fibres were more intensely stained than any other structure in that part of the hind brain. It was therefore possible to trace these fibres back in serial frozen sections to their cells of origin which were found to lie in two discrete nuclei astride the genu of the facial nerve (*Figure 2.5*)[60,61]. These histochemical findings were much more consistent with the results of electrophysiological experiments than any of the earlier attempts with histological methods, most of which had proved unsatisfactory. Interesting results have also been obtained in other parts of the brain. This technique provides a valuable method of distinguishing and delineating nerve nuclei, and sometimes gives a clue to their possible function. It has also revealed a secondary component in a number of important fibre tracts previously assumed to be homogeneous.

One very recent development is the combining of this technique with operative procedures. A serious difficulty in studying nerve pathways which both originate and terminate within the brain is that of discovering in which direction the fibres go. However, if the hypothesis of axonal flow is correct, one might expect that interrupting a cholinergic nerve would cause a piling-up of enzyme on the cell-body side and possibly a loss on the distal side of the interruption. The histochemical results of such an experiment are as predicted and whether or not the hypothesis is correct this procedure provides an excellent method of determining the direction of fibre tracts. The most striking histochemical picture is obtained about 4 days after the operation. On the proximal side of the lesion, the fibres are dilated and very heavily stained for a distance of about 2 mm, whereas on the distal side, staining is much less than normal and may disappear altogether (*Figure 2.5*). Preliminary work with this technique has revealed, among other things, a massive afferent supply to the hippocampus from the septal region travelling in the cingulum, fimbria and dorsal fornix, for which there was little previous anatomical evidence though some from electrophysiology. Further work with smaller, accurately-placed lesions should provide a wealth of new information about fibre connexions in the central nervous system.

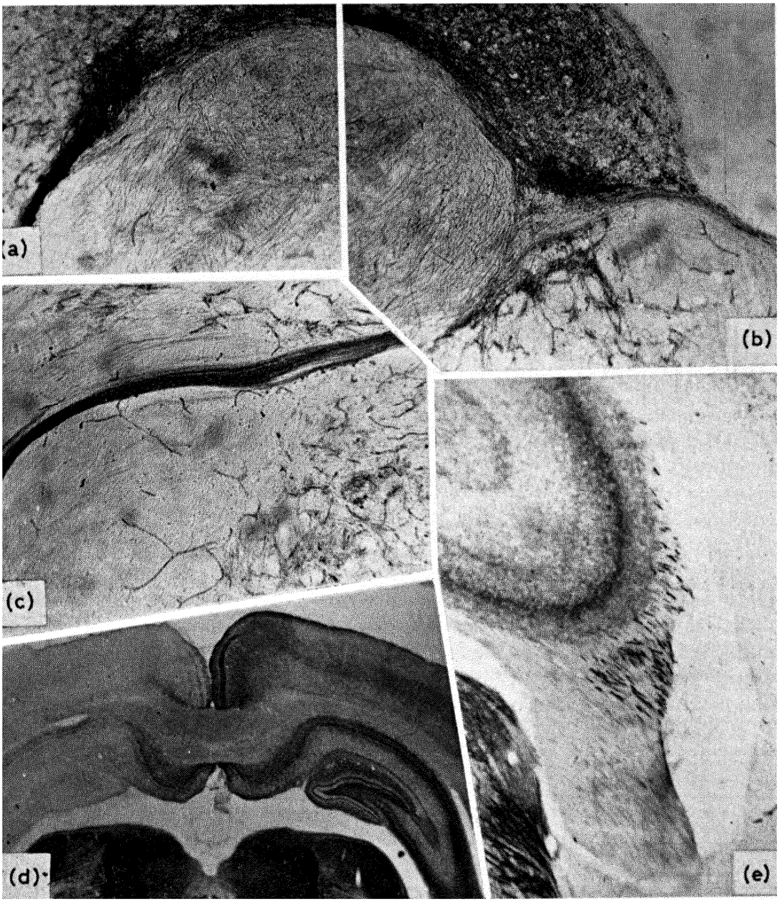


Figure 2.5. Photographs[60,61] of transverse frozen sections of rat brains stained by the thiocholine technique at a pH of 4·7 to 5·0
(*a*) Lateral and (*b*) medial salivatory nuclei as heavily stained, compact groups of cells on either side of the facial genu. (*c*) A heavily stained bundle of salivatory fibres traversing lateral reticular formation and arching over spinal trigeminal tract. (*d*) Loss of staining of hippocampal formation and cingulate gyrus on left-hand side in a rat killed 8 days after total interruption of left fimbria and alveus. (*e*) Normally-stained fibres in the lower part of the fimbria and altered fibres with enzyme pile-up in the upper part rostral to a partial lesion made 4 days beforehand. ((d) and (e) by courtesy of S. Karger, Basel/New York)

THE FUTURE

Histochemistry has grown so rapidly that the development of new and improved techniques has advanced much faster than the ability of biologists to make full use of them. Thus our knowledge of the chemical composition of cells would continue to increase rapidly for many years even if no further technical advances were made. Also, many biologists have been slow to

appreciate the potential value of histochemistry in their own particular fields, not as an end in itself but as a new means of approach to the solving of old problems. We can therefore consider the future of histochemistry under three broad headings: development of new techniques, study of new tissues, and use of histochemistry in other fields of biology.

The present techniques for nucleic acids are probably adequate for most studies with the light microscope and it seems unlikely that any new chemical methods will have much effect in this field. General methods for carbohydrates are also good but the range of compounds of this class is so wide that one could wish for some auxiliary techniques with a narrow specificity. The introduction of oxidizing agents which are more selective in their action than periodic acid should be a promising line of approach. The use of lead tetra-acetate in the presence of boric acid, which chelates with *cis*-glycols, is an example of the type of development to be expected, although this particular one has not proved as satisfactory as was hoped[62]. A most disappointing fact is that although the use of enzymes for selective removal of particular cell components is a very powerful method of improving the specificity, only two (ribonuclease for RNA and diastase or amylase for glycogen) have proved satisfactory in practice. This list may be rapidly extended, however, as a result of recent developments in methods for the separation of pure enzymes.

Methods for lipids and proteins have not been specifically considered. Here the present situation is less satisfactory but prospects for the development of new or greatly improved techniques are good since there are so many reactive groups which may be present. The *pseudo*-isocyanine technique[13] for insulin is an example of what can be confidently expected. A major limitation of present techniques is their low sensitivity in thin sections. This could be overcome by the general introduction of fluorescent techniques which have a far higher potential sensitivity since the reactive substance is detected by the emission of light rather than by its absorption. There are serious chemical and physical problems associated with such techniques but these should eventually be solved. At present only about 10 per cent of the enzymes catalogued by biochemists can be studied by histochemists but the percentage increases year by year and will continue to do so.

These are some of the advances likely in the next few years. The more distant future is difficult to predict, but three branches of histochemistry seem destined to become important eventually: radioautographic, electron microscope and fluorescent antibody techniques. They have not been discussed in this review because they are in the early stages of development where each new research problem tends to raise fresh experimental complications, and it may take many years for them to reach the stage of being suitable for routine application. The resolving power of radioautographic techniques is likely to remain poor and the preparation of pure antibodies tedious; so electron microscopy seems the most promising of the three. Some success has been achieved with quantitative histochemical techniques, particularly in the study of nucleic acids. There are many difficulties in obtaining accurate results, however, and so much can still be learnt from qualitative or semi-quantitative methods that quantitative histochemistry is likely to remain a specialized field for some time to come.

As far as the study of new tissues is concerned, it is only necessary to point out that although histochemists have ranged far and wide, there are still many tissues which have not been adequately studied in mammals and even less work has been done in other animal groups.

The use of histochemical techniques as a routine tool in subjects such as physiology and embryology is only just beginning. As their use increases, however, they are bound to provide a rich harvest of knowledge. They will be of obvious value where there is a specific test for a substance of known physiological function, *e.g.* for endocrine secretions and enzymes such as cholinesterase, or where they can be used to distinguish different cell types and cells in different states of activity. Their less obvious, and much greater value will only be realized when histochemistry is fully incorporated into that body of knowledge which a research worker in any branch of biology must be aware of. It is for this reason that one hopes that histochemistry will not become a separate independent discipline, but will become part of 'what every good biologist should know'.

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3

PROTEIN BIOSYNTHESIS IN MICRO-ORGANISMS

H. TRISTRAM

INTRODUCTION

WITHIN the last twenty years considerable progress has been made in understanding the mechanism and control of protein synthesis. This progress can be attributed to the fruitful co-operation of workers interested in differing fields of biology, and has been stimulated by the realization that macromolecules play a prime part in the growth and reproduction of living matter. The demonstration that deoxyribonucleic acid (DNA) is, in certain viruses, bacteria and probably in all organisms which contain it, the carrier of genetic information, that the information consists largely of amino acid sequences in proteins, and that alteration in genetic material (mutation) leads to the appearance of proteins altered, either biologically or structurally, has shown geneticists and biochemists studying protein synthesis that they are studying different aspects of what is basically the same phenomenon. The pioneer work of Brachet and Caspersson led to the realization that ribonucleic acid (RNA) is implicated in the synthesis of protein, so linking the three types of macromolecule common to almost all living material. The study of the physical and chemical characteristics of biological macromolecules has also interested chemists and biophysicists and considerable progress has been made in elucidating the structural complexity of nucleic acids and proteins. An important factor in making these advances possible is the development of techniques capable of separating minute quantities of material and determining the characteristics of the fractions obtained. Important among these techniques are chromatography, electrophoresis, counter-current distribution, immunological techniques, x-ray analysis, ultracentrifugation, the use of radioactive tracers and the procedures available for the determination of amino acid sequences in proteins.

Proteins catalyse, in a highly specific way, the hundreds of reactions involved in the biosynthesis of cell constituents and in the production of metabolic energy to drive these biosyntheses. Basically all proteins are polymers of some twenty L-amino acids, and owe their high degree of enzymic specificity and their marked differences in physico-chemical behaviour to the specific arrangement of the amino acids within the molecule and to folding and cross-linking between the folds[1]. The amino acids are linked in specific sequence by α-peptide linkages involving the carboxyl and amino group of the α-carbon atom of each amino acid.

The backbone of amino acid residues, which may run to several hundreds in a single protein molecule, is termed the *primary structure*. Such a molecule, in solution, would assume a random coil configuration. Most proteins do not behave as random coils, but assume an ordered spatial structure due to

folding or formation of helical regions as a result of hydrogen bond formation. This stabilizing configuration is known as *secondary structure.* A superfolding also exists superimposed on the secondary structure and is known as the *tertiary structure*, this stability being due to electrostatic and other non-covalent bonds[1,2].

Lastly must be mentioned the occurrence of disulphide bridges (—S—S—) formed between pairs of cysteine molecules, together with a variety of other covalent bonds which form fixed cross-linkages either between adjacent folds or between the individual polypeptide chains of a compound protein. Many workers[2,3] regard these cross-links as part of the primary structure, but in a recent publication Linderstrøm-Lang, who first proposed this terminology, regarded them as part of the tertiary structure[1].

The study of the complex process of protein synthesis poses many questions, the most important being:

(*a*) How is the information concerning the specific amino acid sequences of proteins stored in the genetic material?

(*b*) How is this information passed to the site of protein synthesis and how are the amino acids ranged in correct sequence?

(*c*) Where is the site of protein synthesis?

(*d*) What is the actual biochemical mechanism by which peptide bonds are formed between neighbouring amino acids?

(*e*) How is the secondary and tertiary structure determined?

(*f*) What are the mechanisms which determine the amount of any specific protein manufactured?

At present a complete answer cannot be given to any of these questions, but recent work has provided a partial answer to many of them.

Ideally the mechanism of protein synthesis should be studied by following the increase of a specific protein in a cell-free preparation capable of separation into defined fractions in order to differentiate those cell components essential for the synthesis of proteins from those which are not essential. With one or two exceptions this ideal has not been attained. Workers in this field have been forced to depend on the determination of the incorporation of radioactive amino acids into a gross protein fraction (obtained as material insoluble in *hot* trichloroacetic acid) as a measure of protein synthesis. Before amino acid incorporation can be equated with protein synthesis a number of established criteria, which need not be detailed here, must be met[4]. In addition, especially in investigations with micro-organisms, increases in the activity of specific enzymes are frequently used for the study of protein synthesis.

THE MECHANISM AND SITE OF PROTEIN SYNTHESIS

The study of mammalian systems *in vitro* has recently contributed much information about the biochemical mechanism of protein synthesis. Although this account is concerned chiefly with micro-organisms it will be convenient to discuss briefly the advances made as a result of the study of mammalian systems and to examine whether, and to what extent, similar reactions

appear to be present in micro-organisms. Recent work with cell-free preparations from mammalian tissues stems from earlier *in vivo* studies in which radioactive ^{14}C-labelled amino acids were fed to animals, followed by homogenization of individual tissues and isolation, by centrifugation, of various cell organelles with the aim of determining the sites of active incorporation into protein. The result of many such investigations was to implicate the microsome as the major site of amino acid incorporation. Microsomal particles were isolated as the fraction of homogenates of mammalian tumour and liver cells sedimented by centrifugation at 100,000 *g* after prior removal of nuclei and mitochondria by low-speed centrifugation, and were studied biochemically long before they were identified and localized visually within the cell. The microsome fraction of tissue homogenates is now known to be derived from the so-called 'endoplasmic reticulum', a lipid-rich, three-dimensional, cytoplasmic membranous structure, parts of which bear electron-dense granules 100 to 150 Å in diameter[5,6]. The microsome fraction, as isolated from homogenates consists of small pieces of reticulum membrane with attached particles. Treatment with sodium deoxycholate results in dissolution of the membrane fragments and the resulting particles can be recovered by centrifugation. Crude microsome preparations contain protein, RNA and phospholipid, the latter and much of the protein being removed by sodium deoxycholate treatment; the resulting particles, consisting of ribonucleoprotein (RNP), are frequently referred to as 'ribosomes'.

In *in vitro* systems under appropriate conditions radioactive amino acids are incorporated into the protein fraction of such microsome preparations. For incorporation to occur it is necessary to incubate together microsomes, the cytoplasmic soluble fraction (cytoplasmic sap), adenosine triphosphate (ATP) and a system for the continuous generation of ATP (generating system). It was subsequently shown that the cytoplasmic sap can be replaced by a fraction precipitated from it by acidification to pH 5 (the pH 5 enzyme fraction) and that incorporation is then dependent on the addition of guanosine triphosphate (GTP), in addition to the compounds already mentioned[4]. The incorporation of ^{14}C-labelled amino acids by rat liver microsomes under these conditions lasts only about 20 minutes and a net synthesis of protein has not been demonstrated, although it has been shown that the amino acids are incorporated into α-peptide linkages.

In 1941, Lipmann, discussing the role of ATP in biosynthetic reactions, suggested that 'activation', probably involving phosphorylation of the α-carboxyl groups of amino acids, might be a necessary preliminary for protein synthesis. Other workers made similar suggestions though did not always invoke the carboxyl group as the site of activation. In 1955 the presence, in the 'pH 5 enzyme fraction' of rat liver, of enzymes responsible for the activation of amino acids was demonstrated[7,8]. The activation, which is dependent on the presence of ATP and magnesium ions, leads to the formation of an amino acyl adenylate and pyrophosphate (PP):

$$\underset{\text{(amino acid)}}{R{\cdot}CH{\cdot}NH_2{\cdot}COOH} + ATP \rightleftharpoons \underset{\text{(amino acyl adenylate)}}{AMP{-}CO{\cdot}CH{\cdot}NH_2{\cdot}R} + PP \quad (3.1)$$

Since amino acyl adenylates cannot be found free in the reaction mixture it

was postulated that they remained bound to the activating enzyme. The activity of these enzymes may be measured in two ways:

(*a*) Since the activation reaction is reversible ^{32}P-labelled pyrophosphate is incorporated into unlabelled ATP in the presence of activating enzyme and amino acid. The stimulation of this incorporation, known as the ATP–pyrophosphate exchange reaction, by amino acids is used as a measure of amino acid activation.

(*b*) In the presence of hydroxylamine an amino acid hydroxamate is formed which, under appropriate experimental conditions, may be estimated colorimetrically:

$$\begin{aligned} &\mathrm{R{\cdot}CH{\cdot}NH_2{\cdot}COOH + ATP + NH_2OH \rightarrow} \\ &\underset{\text{(amino acid hydroxamate)}}{\mathrm{H_2N{\cdot}R{\cdot}CH{\cdot}CO{\cdot}NHOH}} + \mathrm{AMP + PP} \end{aligned} \tag{3.2}$$

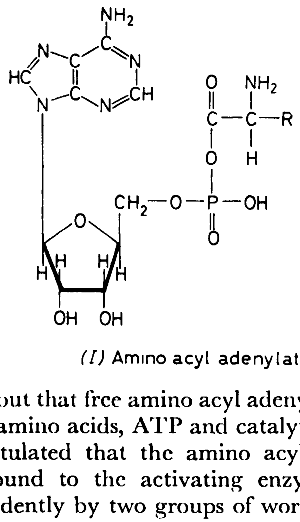
Following the discovery of amino acid activation in mammalian cells, the presence of similar enzymes was demonstrated in a wide variety of bacteria, including *Escherichia coli*, *Proteus vulgaris*, *Rhodospirillum rubrum* and a number of lactic acid bacteria, and the fungi *Neurospora crassa* and *Saccharomyces cerevisiae*[7-11].

Many earlier investigations were conducted with a complete mixture of naturally-occurring amino acids, but when single amino acids were added to a cell-free preparation containing activating enzymes only eight to ten amino acids appeared to be activated, as measured by the ATP–pyrophosphate exchange reaction. If amino acid activation is an essential step in protein synthesis, then it would be expected that all amino acids should be capable of activation. The failure to observe activation of some of the amino acids common in proteins could mean that activation is not universal to all amino acids (and consequently probably not involved in protein synthesis) or that specific activating enzymes exist for each amino acid, some being more labile than others, their activity being lost during preparation of the cell extract. Other factors, which need not be discussed here, could also lead to the failure to observe stimulation of the ATP-pyrophosphate exchange reaction with some amino acids[7]. More recently preparations capable of activating all twenty naturally-occurring amino acids have been obtained from animal tissues, higher plant tissues and bacteria[8]. Subsequent investigations led to the isolation, from crude extracts, of fractions having high activity towards a single amino acid. Purified amino acid activating enzymes have now been obtained for about half the amino acids commonly found in proteins, a number of these being obtained from micro-organisms[8,12]. It would appear likely that enzymes responsible for the activation of all amino acids occurring in proteins will ultimately be isolated. With few exceptions amino acid activating enzymes are highly specific for a single amino acid. One enzyme isolated from *E. coli* activates both valine and isoleucine, although the relative affinity of the enzyme for the two substrates varies[12].

Most amino acid activating enzymes activate only the L-isomers, *i.e.* the form in which amino acids occur in proteins. D-isomers tend to inhibit the activation of the corresponding L-isomers in *E. coli* extracts[13,14], but other workers have not confirmed this result[9]. However, partially purified extracts of *Lactobacillus arabinosus* and certain other bacteria catalyse the formation of

an amino acid hydroxamate in the presence of D-alanine[8]. The significance of this observation may reside in the fact that D-alanine is a constituent of cell wall peptides in many bacteria, and suggests that incorporation into such peptides may require prior activation of the amino acids. Glycine appears to be activated by a mechanism differing from that described above, at least in *Photobacterium fischeri*[8].

Reversibility of reaction (3.1) was established by the demonstration that incubation of synthetic amino acyl adenylates with pyrophosphate and a suitable amino acid activating enzyme results in the formation of ATP[8,10]. Although activating enzymes are usually highly specific in the forward reaction, they are surprisingly unspecific in catalysing the breakdown of amino acyl adenylates[10,15]. For example, a yeast preparation capable of activating L-methionine formed ATP, not only from L-methionyl adenylate, but also from D-methionyl, L-seryl and L-phenylalanyl adenylates[10].



(I) Amino acyl adenylate

It has been pointed out that free amino acyl adenylates cannot be detected in systems containing amino acids, ATP and catalytic amounts of activating enzymes. It was postulated that the amino acyl adenylates (*I*) formed remained strongly bound to the activating enzymes, a suggestion now demonstrated independently by two groups of workers[15,17]. By using large amounts of purified tryptophan activating enzyme from beef pancreas in the presence of magnesium ions, radioactive α-^{32}P-ATP and ^{14}C-labelled tryptophan, followed by incubation and removal of enzyme protein with trichloroacetic acid (TCA), it was shown that the resulting supernatant contained a product possessing properties of an authentic sample of tryptophanyl adenylate. This material was removed from the activating enzyme during TCA treatment, but could also be removed by hydroxylamine, yielding ^{14}C-labelled tryptophan hydroxamate. Furthermore incubation of the material removed by TCA with more activating enzyme and pyrophosphate resulted[17] in the formation of ATP, which was labelled with ^{32}P.

The pH 5 enzyme fraction of mammalian cells, besides containing amino acid activating enzymes, also contains a certain amount of RNA. It was discovered independently by two groups of workers that incubation of pH 5 enzyme fraction of rat liver or mouse ascites tumour cells with magnesium ions, ATP and ^{14}C-labelled amino acids leads to binding of amino

acids into a fraction insoluble in *cold* TCA, but soluble in *hot* TCA, and this binding is abolished by ribonuclease, *i.e.* the incorporation of amino acids is consistent with the idea that they are bound to RNA. A similar transfer of labelled amino acids to RNA occurs in *E. coli*, yeast, the protozoan *Tetrahymena* and rabbit reticulocytes[4].

The acceptor RNA possesses properties which differentiate it from the bulk of the cell RNA. Quantitatively it forms a relatively small proportion of the total RNA of the cell, although the precise proportion varies in different organisms. It occurs in the cytoplasmic 'soluble' fraction, that is, in the supernatant after removal of the ribosomes (which contain the bulk of the cytoplasmic RNA) by centrifugation at 100,000 *g*, and is known as soluble RNA (s-RNA). More recently, as a result of the biological activity of this RNA fraction, Schweet has suggested the term 'transfer RNA'. The enzymes responsible for activation of amino acids also mediate the transfer of the products of activation to s-RNA[8,12]. The addition of a single ^{14}C-labelled amino acid allows the transfer of a certain amount of radioactivity to s-RNA and this is increased by addition of more s-RNA. Furthermore, the labelling of s-RNA by a single ^{14}C-labelled amino acid is not decreased by the presence of a complete mixture of unlabelled amino acids. Finally the incorporation of radioactivity into s-RNA in the presence of several labelled amino acids is roughly additive[4,8]. These results suggest that either a given s-RNA molecule possesses a limited number of specific sites for the binding of individual activated amino acids, or that the preparations are heterogeneous, consisting of a population of s-RNA molecules, each having a specific binding site (or sites) for a single amino acid species. The latter explanation is probably correct, for s-RNA preparations, isolated from cytoplasmic sap by treatment with phenol or 1 M sodium chloride can be separated into fractions having an enhanced ability to bind a particular amino acid. These procedures have so far achieved only a partial separation, but there seems little doubt that individual amino acids are bound to specific s-RNAs. Animal, bacterial and yeast s-RNA preparations have been studied in this way. The methods used in attempts to separate the individual fractions cannot be discussed in detail, but include counter-current distribution and column chromatography on a variety of materials, both with and without prior chemical modification of the s-RNA[4,8,18,19].

Mammalian pH 5 enzyme fraction preparations, on ageing, tend to lose the ability to bind labelled amino acids, a process accelerated by incubation with pyrophosphate in the absence of added amino acids. The ability of s-RNA to incorporate ^{14}C-labelled amino acids can be restored by incubation (in the presence of pH 5 enzyme fraction) with cytidine triphosphate (CTP) and ATP. Incorporation of ^{14}C–CTP is rapid and independent of added ATP, but rapid incorporation of ^{14}C–ATP is dependent on the presence to CTP. Addition of ^{14}C–ATP and ^{14}C–CTP together reveals that the labelling of s-RNA is additive under these conditions. Cytosine and adenine nucleotides are incorporated into s-RNA in the ratio of about 2:1. Available evidence suggests that the nucleotides incorporated into s-RNA, previously incubated until it no longer accepts amino acids, constitute a terminal group of three nucleotides, two derived from CTP and one from ATP, the two cytosine nucleotides being attached to the s-RNA molecule and the adenosine

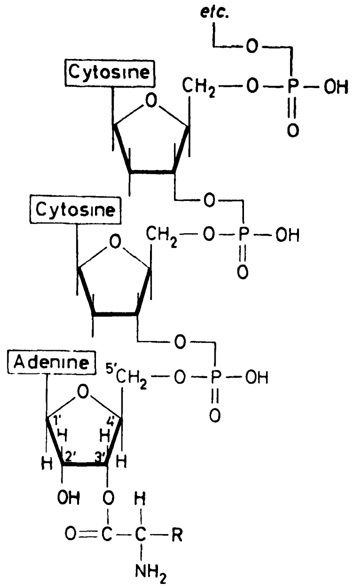
attached terminally to the cytosine nucleotides[4]. Attachment of cytosine nucleotides and adenosine to s-RNA is enzymic and is accompanied by liberation of pyrophosphate. The enzyme responsible, located in the cytoplasmic sap, has been partially purified[4,20]. The nucleotide base distal from the cytidylyl-cytidylyl-adenosine terminal sequence is guanine[21,22]. Incorporation of ^{14}C-ATP into terminal positions of s-RNA of *E. coli* has been demonstrated[23-25], a two to five-fold acceleration of incorporation being apparent in the presence of CTP[25]. Treatment of intact cells of yeast with phenol yielded a nucleic acid preparation which possessed the biological and chemical properties of an s-RNA fraction prepared by more laborious methods involving rupture of the cells[18]. This preparation represents a readily available bulk source of s-RNA, and besides accepting activated amino acids also incorporates radioactivity from ^{14}C-ATP.

The removal of the terminal group of nucleotides from s-RNA leads to inability to bind amino acids unless CTP and ATP are added, together with magnesium ions, activating enzymes and the fraction responsible for attachment of the terminal nucleotide group. Amino acids attached to s-RNA can be readily removed by treatment with alkali at room temperature. Further investigation has shown that treatment of s-RNA previously labelled with ^{14}C-labelled leucine with ribonuclease yields the ^{14}C content quantitatively as ^{14}C-labelled leucyl adenosine[4]. In addition, the radioactivity is recovered as an amino acid–adenosine derivative *irrespective of which amino acid is used.* This is an important observation, since it suggests that all activated amino acids are attached to adenosine in s-RNA. The precise nature of the linkage binding activated amino acids to s-RNA has also been investigated, using systems derived from *E. coli*[26] and from mammalian tissues[4]. Amino acids are attached to the 2′- or 3′-hydroxyl group (precisely which of these is uncertain) of the ribose moiety of the terminal adenosine of s-RNA. The transfer of the amino acid moiety of an amino acyl adenylate (activated amino acid) to s-RNA is accompanied by the release of adenosine monophosphate (AMP). The structure of the terminal portion of s-RNA carrying a 'bound' amino acid is shown in formula *(II)*.

The s-RNA of *E. coli* represents about 10 per cent of the total RNA of the cell. The molecular weight, established by a variety of methods[8,25,27,28], has been variously estimated at between 25,000 and 30,000. After alkaline hydrolysis of *E. coli* s-RNA about 95 per cent of the nucleoside residues liberated (representing the terminus to which activated amino acids become attached) are accounted for as adenosine, an observation in good agreement with the results discussed above. One adenosine residue for each 80 to 100 nucleotides appears in such hydrolysates[24,25,27].

Evidence has been obtained that the s-RNA of *E. coli* is not combined with protein in the cytoplasm, but exists in the free state and possesses a secondary helical structure due to hydrogen-bonded base pairing, not as in DNA, between two different strands, but between portions of the same molecule[28,29]. Apart from differences of molecular weight, s-RNA differs from ribosomal RNA in its base composition. The cytosine content of *E. coli* s-RNA is considerably higher and that of uracil is lower than the corresponding amounts in ribosomal RNA[4,27]. In addition, the s-RNA of

E. coli, yeast and rat liver contains larger proportions of 'atypical' bases (*i.e.* bases other than uracil, cytosine, adenine and guanine) than do the corresponding particulate RNAs. In particular, *pseudo*-uracil (5-ribosyl uracil) occurs in considerable amounts in s-RNA from all three sources, but is practically absent from ribosomal RNA. In addition s-RNA contains



(II)

appreciable amounts of methylated purines[4,8,12,27,30]. It has been shown that, in yeast, the presence of *pseudo*-uracil seems to be confined to the RNA specifically concerned with the transfer of activated amino acids[30].

If activated amino acids are, in fact, bound to specific s-RNA molecules, each of which terminates in a cytidylyl–cytidylyl–adenosine sequence and a guanine residue at the distal end, it would appear that the specificity of s-RNA resides in the remainder of the molecule, a sequence 80 to 100 nucleotides long. Furthermore, if, as evidence already discussed suggests, the products of amino acid activation remain strongly bound to the surface of the activating enzymes, it is probable that 'recognition' of a specific s-RNA by its activated amino acid resides in the enzyme molecule, which may possess, besides the active centre involved in the formation of the amino acyl adenylate, a site capable of 'recognizing' and orientating the s-RNA species specifically competent to accept the product of the particular activating enzyme. Preliminary studies have established that a s-RNA preparation from *E. coli* is heterogeneous with respect to its sequence of bases[31] and that very slight modification of the base sequence by the action of nitrous acid

(see p. 131) leads to loss of ability of s-RNA to accept activated amino acids from the activating enzyme[21].

Mammalian s-RNA, 'loaded' with a radioactive amino acid as described, can transfer amino acid to isolated microsomes where the radioactivity is incorporated into protein. The presence of magnesium ions, ATP, ATP generating system, GTP and an enzyme component of the cytoplasmic sap fraction are all necessary for efficient transfer[4]. The presence of GTP is essential for the over-all incorporation of amino acids into microsome protein, but its role is unknown. It is not required for amino acid activation or for transfer of amino acyl adenylates to s-RNA. The enzymic component necessary for transfer has been partially purified from mammalian sources[32], higher plants[33] and *E. coli*[34]. *In vivo* kinetic experiments with mammalian cells have established that labelled amino acid passes through s-RNA-amino acid complexes before being incorporated into protein, so providing strong evidence for the belief that RNA-bound amino acids constitute obligatory intermediates in the biosynthesis of protein[4]. This conclusion is further supported by the observation that addition of labelled amino acids to growing cultures of *E. coli* results in the formation of complexes between the amino acids and RNA, the properties of the complexes being identical with those of the s-RNA–amino acid complexes formed in *in vitro* systems[35]. Radioactivity found in this RNA fraction after brief exposure to labelled methionine decreases rapidly on addition of excess unlabelled methionine, the displacement giving rise to an almost equivalent increase in the radioactivity of the protein fraction. These results can be taken to indicate that in intact cells amino acids are transitorily bound to RNA before entering into stable peptide linkage in proteins.

If, as already suggested, the site of protein synthesis in mammalian cells resides in the ribosomes, and activated amino acids are conveyed to the site by s-RNA, then it is of interest to determine whether s-RNA becomes associated with microsomes. Although the process is by no means understood, experiments with labelled s-RNA show that such an association does occur[4,36,37]. On the basis of kinetic experiments using s-RNA (labelled with ^{32}P) carrying activated ^{14}C-labelled valine it has been stated that binding of amino acids into peptide linkages requires 'a large fraction of the associated s-RNA molecule to become firmly and transitorily bound to microsomal RNA'[37]. The overall mechanism of protein synthesis emerging from the investigations described is shown diagrammatically in *Figure 3.1.* Amino acids (AA) react with activating enzymes, yielding amino acyl adenylates (AMP—AA) which are transferred directly from the activating enzyme surface to s-RNA having specific nucleotide sequences terminating in cytidylyl–cytidylyl–adenosine (s-RNA·CCA), AMP being liberated in the process. Under the influence of GTP and a cytoplasmic enzyme fraction amino acids are transferred to the site of protein synthesis on the ribosome, the s-RNA becoming associated with ribosomal RNA in the process. All the amino acid molecules necessary for synthesis (AA_1, AA_2, AA_3 *etc.*) are conveyed to the site in this way and, in a manner not yet understood, the peptide linkages between adjacent amino acids are formed, a process followed by 'stripping' of the newly synthesized protein from the ribosome. The s-RNA is released probably in an unaltered state[34], though whether

before or after the formation of peptide linkages between the amino acids, is not known. For reasons which will become apparent later, liberation of s-RNA *after* peptide bond formation seems more likely.

The scheme shown in *Figure 3.1* is based largely on observations made originally with animal systems. Studies with micro-organisms have, in the main, substantiated this hypothesis. For example micro-organisms possess amino acid activating enzymes and s-RNA having specificities and properties comparable with those of mammalian cells. However, although bacteria

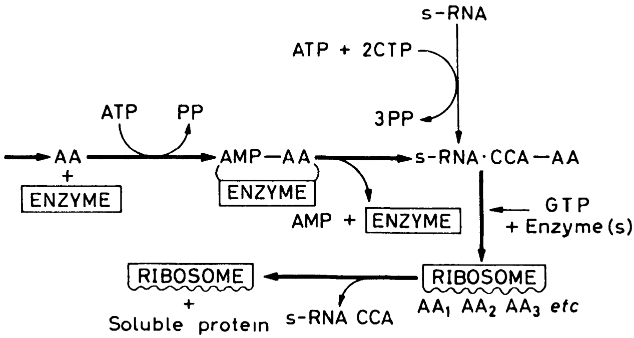


Figure 3.1

contain ribosomes earlier work suggested that RNP particles may not be the site of protein synthesis in these organisms. Furthermore claims have been made for alternative pathways of amino acid incorporation into protein in bacteria. These aspects will be discussed in greater detail in succeeding sections.

The Site of Protein Synthesis in Bacteria

Yeast and bacterial cells contain RNP particles which closely resemble those present in higher plant and animal cells[16]. The majority of bacterial ribosomes, unlike those of mammalian liver cells, lie freely in the cytoplasm. In this respect they resemble those of certain plant and animal cells which appear to lack an organized endoplasmic reticulum[38].

The ribosomes of *E. coli* contain about 60 per cent RNA and 40 per cent protein[39], compared with the 40 per cent RNA and 60 per cent protein found in ribosomes of yeast, pea seedlings and liver[4]. In addition, small amounts of polyamines have been found in *E. coli* and animal ribosomes, although it is not certain that these form an integral part of the particles. Metal ions, particularly magnesium ions, are also present[40].

Examination of cell extracts in the ultracentrifuge has revealed the presence of distinct classes of RNP particles, distinguishable by differences in sedimentation rates. Investigation of the effects of divalent metals on ribosomes of pea seedlings, yeast and liver showed that the larger particles can dissociate into smaller units and that these re-associate under the influence of changes in magnesium ion concentration. The magnesium ion is associated almost exclusively with the RNA component of the particles. In addition to magnesium, calcium ions also appear to be necessary for the

stability of pea seedling ribosomes[4,16,40]. The ribosomes of the bacteria so far examined display a similar phenomenon, the effect of magnesium ion concentration on the RNP particles of *E. coli* having been examined in detail. Preparation of a bacterial extract in the presence of 0·01 M magnesium acetate yields mainly particles of 100 S (Svedberg units, a measure of rate of sedimentation in the ultracentrifuge), which dissociate into 70 S particles on lowering the magnesium ion concentration to 0·001 M. A further decrease in magnesium ion concentration results in the appearance of a mixture of 50 S and 30 S particles. The process of dissociation can be reversed by appropriate increases in magnesium ion concentration[39]. The results of this investigation may be summarized in the following way:

$$30\,S + 50\,S \rightleftharpoons \begin{matrix} 70\,S \\ + \\ 70\,S \end{matrix} \rightleftharpoons 100S$$

Molecular weights: $0{\cdot}7 \times 10^6$ $\quad 1{\cdot}8 \times 10^6$ $\quad 2{\cdot}6 \times 10^6$ $\quad 5{\cdot}4 \times 10^6$

—Increasing Mg^{++} concentration →

The 70 S particle is composed of one 30 S and one 50 S particle and the association of two 70 S particles yields a single 100 S particle. Electron microscopy of ribosome preparations has elegantly confirmed these conclusions. The 70 S particles are seen to consist of two sub-units, one of which is more or less spherical and resembles closely the appearance of a 50 S particle; the other sub-unit is markedly asymmetrical and resembles a 30 S particle in appearance. The 100 S particles are revealed as associations of two 70 S units, the two larger sub-units of the 70 S particles being adjacent and the two smaller sub-units opposed[41,42]. More recently it has been shown that a certain proportion of the larger ribosomes of *E. coli* do not reversibly dissociate under the influence of changes in magnesium ion concentration[43,44].

The relative proportions of particles of differing sizes found in *E. coli* cells vary with the growth phase of the culture. During the exponential phase 70 S to 85 S particles predominate, with smaller amounts of 50 S and 30 S and virtually no 100 S particles. Within a few minutes of cessation of exponential growth due to exhaustion of glucose 100 S particles predominate, followed by a rapid reversion to the pattern characteristic of exponentially growing cells on addition of more glucose. Exhaustion of magnesium ions from the medium also has a profound effect on the ribosomal population of *E. coli* cells[45].

The isolated RNA of ribosomal particles from various sources consists of single-stranded molecules containing double helical sections, formed as a result of hydrogen bond formation between bases of adjacent portions of the same molecule. The helical sections probably alternate with 'looped out' portions lacking hydrogen bonds[46,47]. Available evidence, based on a comparison of the behaviour of isolated ribosomal RNA and intact ribosomes of *E. coli*, suggests that the structure of the isolated material is comparable with that of *in situ* RNA[48,49]. RNA isolated from ribosomes by comparatively mild procedures contains two components possessing sedimentation coefficients of about 16 S and 25 S. The 30 S ribosomes of *E. coli* contain only 16 S RNA, while 50 S and 70 S ribosomes yield a mixture of 16 S RNA and

25 S RNA[50]. The base composition of RNA from *E. coli* ribosomes of differing sizes is virtually identical[4,51] and differs strikingly from that of s-RNA. The study of the proteins of ribosomes has scarcely begun, but they are known to contain only small amounts of sulphur-containing amino acids and to be rich in arginine and lysine. Certainly there is marked heterogeneity of ribosomal protein, but no outstanding differences have been detected between preparations from different organisms[4,40,52,53]. Ribosomes of *E. coli* contain deoxyribonuclease and ribonuclease, the latter also being present in ribosomes of higher organisms[16,54]. These enzymes probably form an integral part of the ribosomes since they are 'latent', *i.e.* their activity is only apparent after treatment tending to separate the RNA and protein moieties of the ribosomes. Many other enzymic properties have been ascribed to ribosomes; the significance of these observations will be discussed below.

Although bacterial cells contain large numbers of ribosomes, earlier studies tended to suggest that the site of protein synthesis in bacteria resided, not in the RNP particles, but in the cytoplasmic membrane. Before discussing the evidence which led to this view it is necessary to give an indication of the methods commonly used for obtaining preparations of cytoplasmic membranes of bacteria.

The enzyme lysozyme digests the cell walls of Gram-positive bacteria such as *Bacillus megaterium* with the resulting lysis of the cell if the digestion is carried out in hypotonic solution. If, however, the suspending medium is made hypertonic by the addition of phosphate, sucrose or other suitable agent, then digestion with lysozyme leads to the formation of spherical, osmotically fragile protoplasts, which disintegrate under the influence of osmotic shock on dilution of the suspension fluid with water. From a suspension of protoplasts treated in this way centrifugation allows the collection of protoplast 'ghosts', which are, in fact, the ruptured cytoplasmic membranes, from which all or most of the cytoplasmic contents have escaped. Although lysozyme alone does not digest the cell walls of Gram-negative bacteria such as *E. coli*, it can be induced to act on these organisms by addition of various substances or by combining lysozyme treatment with freezing and thawing. A variety of other methods, including exposure to penicillin, allow the preparation of osmotically sensitive spheres from *E. coli*[55,56]. Since there is no evidence that any of these osmotically fragile bodies derived from Gram-negative organisms are completely devoid of wall material the term 'spheroplast' has been suggested to describe such preparations.

Exposure of intact cells of *Bacillus megaterium* to ^{14}C-labelled amino acids, followed by fractionation by exposure to lysozyme, osmotic lysis of the resulting protoplasts and differential centrifugation of the lysates revealed the heaviest labelling in the cytoplasmic membrane fraction in experiments of a few minutes' duration. Similar results were obtained after exposure of whole protoplasts or isolated protoplast membranes to labelled amino acids[57,58].

An investigation of ^{14}C-labelled leucine incorporation into protein of intact spheroplasts of *E. coli*, obtained by the penicillin method, also led to the conclusion that the membrane fraction is the main site of amino acid

incorporation into protein in this organism[59]. Isolated membranes also actively incorporated ^{14}C-labelled leucine into protein. Further evidence suggesting that the cytoplasmic membrane is more active than ribosomes in amino acid incorporation in bacteria has been presented for *E. coli*, *Bacillus subtilis* and *Azotobacter*. These investigations are described in detail in a recent review[8].

Pioneer work on the incorporation of radioactive amino acids, mainly glutamate, into the TCA-insoluble material of subcellular preparations of *Staphylococcus aureus* was carried out by Gale and Folkes, using ultrasonically-treated cells[60]. This preparation consists of cells, the outer envelopes of which have been ruptured, and contains wall, membrane, some residual cytoplasmic material and probably nuclear material. Although amino acids are incorporated into the wall of intact cells of this organism, incorporation into this fraction is small after ultrasonic treatment, and incorporation into TCA-insoluble material other than wall components can be demonstrated under appropriate conditions. It seems likely that it is the membrane which is active in this respect.

A recent important contribution, besides demonstrating the importance of ribosomes in protein synthesis in *E. coli*, suggests an explanation of observations indicating that the cytoplasmic membrane is the site of protein synthesis in bacteria. The incorporation of ^{35}S-labelled sulphate into the proteins of *E. coli* was investigated in experiments of a few seconds' duration[61]. Sulphur was chosen as the radioactive tracer to prevent difficulties of interpretation due to incorporation of radioactivity into the *structural* proteins of the RNP particles since the content of sulphur-containing amino acids of the ribosome protein is very low[16]. Furthermore, synthesis of structural proteins is reduced to a minimum in these short-term experiments.

Growing cells, after exposure to ^{35}S-labelled sulphate, were ruptured and fractionated by centrifuging in a sucrose gradient which allowed the collection, without mixing, of the particulate fractions of varying sedimentation rates. In short experiments (5 to 20 seconds) most of the radioactivity was located in the 70 S to 85 S particles, but in longer experiments (45 to 120 seconds) the specific activity of the 50 S particles increased and surpassed that of the large particles. By means of 'pulse' experiments entailing exposure of cells to ^{35}S-labelled sulphate for 15 seconds followed by a non-radioactive 'chaser' of unlabelled sulphate plus unlabelled sulphur-containing amino acids to dilute out the unincorporated tracer, it was demonstrated that radioactivity incorporated in the 70 S particles could pass, within a few seconds, into the soluble fraction (*Figure 3.2*). The increase in the radioactivity of the soluble protein fraction was roughly equivalent to the decrease in radioactivity of the ribosomes.

It was concluded that there is 'a protein component which is transiently associated with the ribosomes and has all the characteristics which would be expected in a compulsory precursor of the soluble proteins'. Similar 'pulse' experiments with a mixture of ^{14}C-labelled amino acids led to essentially similar observations. The material attached transitorily to the ribosomes was referred to as 'nascent' protein. The demonstration that the label resided in cysteine and methionine (both sulphur-containing amino acids) bound by peptide linkages and that partial hydrolysis yielded a large

number of peptides labelled with ^{35}S established that the radioactive material associated with the ribosomes was, in fact, protein in nature[45,61].

Although the fraction containing fragments of cell wall and cytoplasmic membrane incorporated ^{35}S no evidence was obtained to support the view that the labelled material constituted a major protein precursor. However if cells were broken by lysozyme-freezing treatment[56] only about 50 per cent of the ribosomes were released, but on subjecting this preparation to shearing forces the remainder were detached from the membrane fragments. The

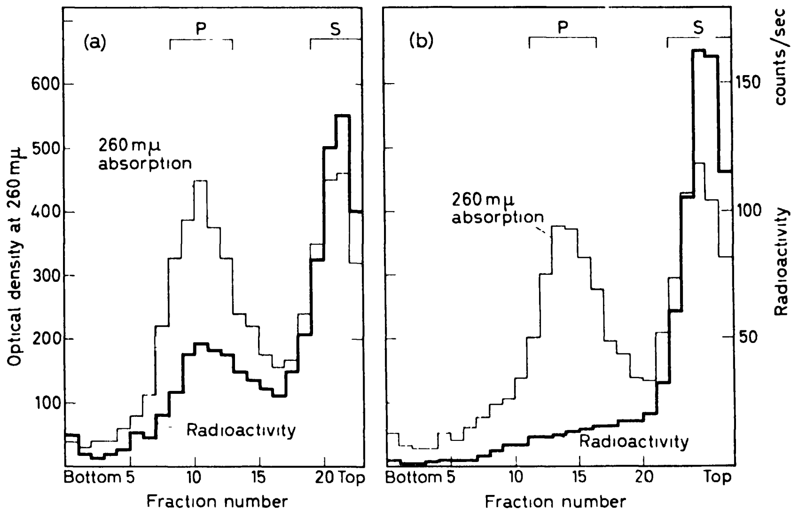


Figure 3.2. Sedimentation analysis of cell juice obtained from growing *E. coli* following (*a*) 15 seconds' exposure to ^{35}S-labelled sulphate and (*b*) 15 seconds' exposure to ^{35}S-labelled sulphate followed by non-radioactive 'chaser' of unlabelled sulphate, methionine and cysteine[61]

Optical density at 260 mμ used to follow distribution of nucleic acid, including that bound in ribonucleoprotein. Note the decrease in radioactivity in particles sedimenting in the 70 S to 85 S region (P) and the increase in radioactivity in soluble (non-sedimenting) protein (region S) following exposure to 'chaser'.

ribosomes liberated by lysozyme-freezing procedure possessed only about 50 per cent of the specific radioactivity found in the ribosomes liberated by shearing forces. It would appear from these observations that, although some ribosomes may occur free in the cytoplasm, others may be more or less firmly bound to the cytoplasmic membrane, the latter being more active in protein synthesis[61].

It is probable that many of the 'membrane fractions' used by different groups of workers, besides being possibly contaminated to an unknown extent by intact cells or protoplasts (see p. 109), contained adhering cytoplasmic material, including ribosomes. That such contamination can occur is suggested by electron microscope studies[62], the apparent variability of membrane fractions prepared by different workers[63] and the fact that, although amino acid activating enzymes are found almost exclusively in the

cytoplasmic sap (100,000 *g* supernatant) in extracts of cells broken by grinding or shaking with glass beads[8], detectable activity is associated with the membrane fractions obtained by the more gentle processes involving lysis of protoplasts (or spheroplasts) or mild ultrasonic treatment[8,58–60,62].

The numbers of RNP particles associated with the membrane may depend on the method used for breaking the cells. This suggestion is supported by electron microscope studies which frequently fail to resolve an inner surface to the cytoplasmic membrane in bacterial cells. There may be no distinct

Table 3.1. Incorporation of ^{14}C-labelled leucine into protein by various fractions from a crude extract of *E. coli*[64]
Each fraction was incubated for 15 minutes at 37°C in the presence of magnesium ions, potassium ions, ATP, ATP generating system, GTP, a mixture of 18 amino acids and ^{14}C-labelled leucine

Fraction	*Total* counts/min	*Specific activity* counts/min/mg protein
1. Crude extract	2,870	757
2. 30,000 *g* sediment	0	0
3. 30,000 *g* supernatant	2,795	777
4. 100,000 *g* sediment	714	340
5. 100,000 *g* supernatant	3	3
6. Fractions 4 + 5 (1.2)	3,000	527

discontinuity between membrane and cytoplasm[63]. It becomes questionable then, whether the particles removed by shearing forces in the experiments discussed above[61] should be regarded as contaminating cytoplasmic particles or an integral part of the membrane. Mitchell, in an excellent review[63] of the structure and properties of bacterial cell organelles states: 'If cytoplasmic constituents adhere to the membrane . . . . in the living cell and are carried with the membrane during its isolation, one may take the view that they should be regarded as integral constituents of the complex sheet defined as the plasma membrane of the living cell'.

A system from *E. coli*, capable of incorporating ^{14}C-labelled amino acids into protein and also bearing a close resemblance to the characteristics of incorporation revealed in mammalian studies, has recently been described[64]. The incorporation of ^{14}C-labelled leucine into *hot* TCA-insoluble material (=protein) by a crude cell extract obtained by grinding cells with fine alumina was dependent on the presence of magnesium ions and stimulated by potassium ions, ATP, ATP generating system, GTP and a complete amino acid mixture. Further fractionation of the crude extract by centrifugation revealed (see *Table 3.1*) that fractions 2 (wall and membrane fragments) and 5 (cytoplasmic sap) were devoid of incorporating activity and that the total activity of the crude extract resided in fraction 3 (ribosomes + cytoplasmic sap). Ribosomes alone (fraction 4) possessed some incorporation activity which could be restored fully by addition of cytoplasmic sap, presumably containing amino acid activating enzymes and s-RNA (*Table 3.1*, line 6). An absolute requirement for GTP was not observed, although its presence was stimulatory. More recently an absolute requirement for GTP for incorporation of amino acids into protein by an *E. coli* ribosome preparation has been demonstrated[65].

An *in vitro* 'pulse' experiment with *E. coli* ribosomes further demonstrated that radioactivity incorporated into the ribosomes could be 'chased out' into the cytoplasmic soluble fraction[43]. Evidence was obtained suggesting that a small proportion (5 to 10 per cent) of the total 70 S ribosomes of the cell become highly radioactive and are relatively stable to lowered magnesium ion concentrations (which cause the dissociation of most of the 70 S particles to smaller entities). This fraction was termed 'active 70 S' particles and may correspond to the active particles apparently attached to the cytoplasmic membrane[61].

Other Incorporation Systems in Bacteria

Gale and his colleagues have studied intensively the incorporation of ^{14}C-labelled amino acids into protein of subcellular preparations, using ultrasonically-treated *Staphylococcus aureus*. Although many of the characteristics of amino acid incorporation by these preparations are consistent with the sequence of events already described, certain observations suggest that differences between the incorporation mechanism in *Staphylococcus aureus* and animal tissues may exist. This account will be confined to these aspects of the incorporation phenomenon which appear to differ from that found in mammalian cells, but this pioneer work with staphylococcal preparations has been described in detail[60].

These preparations, if incubated with a suitable energy source and radioactive glutamate (condition 1), incorporate the amino acid into an acceptor from which it is partially removed by incubation with unlabelled glutamate and an energy source. The material bearing glutamate has been characterized electrophoretically and shows some of the properties of s-RNA, in that formation of the complex is insensitive to chloramphenicol and the glutamate is removed from the acceptor material by mild alkali. On the other hand, the glutamate complex formed under condition 1 differs from amino acid-charged s-RNA in that only 30 to 40 per cent of the amino acid is removed by ribonuclease and the complex is soluble in cold TCA, 60 per cent ethanol and exhibits different behaviour in aqueous phenol. The glutamate released chemically from the acceptor consists of about 50 per cent L-glutamate, the remainder being present as the D-isomer. The significance of this observation may reside in the fact that D-glutamate is a constituent of cell wall peptides in *Staphylococcus aureus*.

If a similar experiment is conducted in the presence of ^{14}C-labelled glutamate and a complete mixture (less glutamate) of unlabelled amino acids (condition 2) the radioactivity is located in material possessing properties of RNP and quite different electrophoretic behaviour from the material responsible for binding of glutamate under condition 1. Labelling a staphylococcal preparation with ^{14}C-labelled glutamate under condition 1, followed by re-incubation with complete amino acid mixture (minus glutamate) leads to a migration of radioactivity from the labelled fraction characteristic of condition 1 to the fraction having the electrophoretic properties of RNP observed in condition 2. Incorporation of glutamate under condition 2 is strongly inhibited by chloramphenicol, known to be a potent inhibitor of protein synthesis, suggesting that incorporation under these conditions represents true protein synthesis.

The partial removal of nucleic acid from ultrasonically-disrupted cells of *Staphylococcus aureus* leads to a diminution in the ability of the preparation to incorporate labelled amino acid under conditions 1 and 2. Re-addition of the extracted nucleic acid or of a nucleic acid digest restores the ability to incorporate amino acids. Material active in stimulating incorporation has been obtained by fractionation of digests of nucleic acid from staphylococcal cells and other micro-organisms. This fraction, termed 'incorporation factor', loses its activity on storage and old preparations contain glycerol. Glycerol itself does not immediately stimulate amino acid incorporation into protein of nucleic acid-depleted disrupted staphylococci, but does so after a lag, when the glycerol (or a derivative of it) is found in an RNA-like fraction containing cytosine and uracil[66].

The precise relationship between 'incorporation factor' and the activated amino acid–s-RNA–ribosome sequence is not understood, but it appears capable of replacing the pH 5 enzyme fraction (activating enzymes plus s-RNA) for the incorporation of amino acids into rat liver microsomes in the presence of ATP, ATP generating system and GTP. Since 'incorporation factor' stimulates incorporation of amino acids by disrupted staphylococcal preparations under conditions 1 and 2 it would appear that the factor acts at an early stage in incorporation, although it is without demonstrable effect on the amino acid dependent ATP–pyrophosphate exchange reaction. The 'incorporation factor' also restores the ability of nucleic acid-depleted staphylococcal preparations to incorporate purines and pyrimidines into nucleic acid, and it has been suggested that its action may lie in promoting formation of nucleic acid of the s-RNA type, though this explanation is not entirely satisfactory[60].

A number of investigators have described systems which appear to incorporate amino acids into protein in the absence of either, or both, amino acid activating enzymes and s-RNA[8,12]. For example a particulate preparation derived from *E. coli*, composed mainly of fragments of cytoplasmic membrane, while apparently lacking the capacity to activate methionine and most other amino acids, nevertheless incorporated methionine into protein[8].

Another particulate preparation, obtained from *Alcaligenes faecalis*, incorporated all L-amino acids into protein. Prolonged washing led to a loss of amino acid incorporation activity, which could be restored by a protein present in the washing fluid and which was later purified. This protein, termed 'amino acid incorporation enzyme', stimulated amino acid incorporation into the protein of the particles, but was devoid of amino acid activating activity (no detectable ATP pyrophosphate exchange reaction), and was presumably devoid of s-RNA.

The purified 'incorporation enzyme' was claimed to replace the pH 5 enzyme fraction in promoting incorporation of ^{14}C-labelled leucine into the protein of rat liver microsomes. More recently it has been shown that highly purified preparations of the 'amino acid incorporation enzyme', besides effecting the incorporation of amino acids into protein, catalysed an exchange reaction between ADP, UDP, CDP, GDP and the corresponding triphosphates. Addition of ATP, GTP, UTP or CTP to a system containing 'amino acid incorporation enzyme' resulted in the liberation of

orthophosphate, a process dependent on the presence of amino acids, each triphosphate reacting with only a specific set of amino acids. The incubation of 'incorporation enzyme', a nucleoside triphosphate and a suitable amino acid mixture led to the formation of peptides[67–69]. The precise significance of these observations and their relationship to protein synthesis is not yet understood.

It has been pointed out[8,12] that claims to have demonstrated amino acid incorporation into protein in the apparent absence of amino acid activating enzymes must be viewed with caution since only very small amounts of such enzymes are necessary for the catalysis of incorporation at the rates normally observed. It is significant that in one such investigation mentioned above methionine, besides being incorporated into protein, was bound in a form soluble in *hot* TCA (suggesting binding to s-RNA), and incorporation into both 'bound' forms was stimulated by ATP. Furthermore well-washed mammalian microsomes and bacterial cytoplasmic membrane fragments frequently contain detectable amounts of amino acid activating enzymes and acceptor RNA. Thus, although the possibility exists that activating enzymes and s-RNA may not be obligatory for amino acid incorporation into protein in some micro-organisms, it cannot yet be accepted that such a situation has been conclusively demonstrated.

A proportion of the labelled amino acids 'bound' by protoplasts of *B. megaterium* is attached to lipid. A 'pulse' experiment involving short exposure of protoplasts to ^{14}C-labelled phenylalanine, followed by unlabelled phenylalanine resulted in a decrease in total radioactivity bound to lipid and a roughly corresponding increase in the protein fraction. It was further claimed that addition of labelled lipid–threonine complex in olive oil solution to protoplasts resulted in passage of radioactivity into protein. Although the last two experiments described show that label in amino acids bound to lipid can be transferred to protein they do not conclusively demonstrate that the lipid–amino acid complexes are obligatory intermediates in protein synthesis[58,70,71]. The suggestion that lipid–amino acid complexes may be involved in protein synthesis has also been made as a result of investigations involving mammalian, higher plant and fungal systems. The evidence for this belief, which cannot be discussed here, is reviewed in the references cited above.

Amino acids have been shown to occur in another type of complex in living cells. These complexes consist of nucleotides or short polynucleotide chains (oligonucleotides) to which are attached either single amino acids or short peptides. Such complexes have been reported in animal tissues, bacteria, yeast and *Chlorella*[11]. More recently the further claim has been made that amino acids enter protein via such peptidyl nucleotidates[72], but the evidence would appear to be capable of alternative interpretations. At present the precise relationship (if any) of lipid–amino acid complexes and peptidyl nucleotidates to protein biosynthesis cannot be determined.

Net Synthesis of Protein and Synthesis of Specific Proteins by Subcellular Preparations

Most investigations on protein synthesis by cell-free preparations from animal tissue and micro-organisms have been based on the incorporation of

radioactive amino acids into protein fractions. Furthermore this incorporation rarely continues for more than short periods, nor does it lead to measurable increases in total protein, *i.e.* net synthesis of protein cannot normally be detected in such experimental systems. In experiments with mammalian microsomes and microbial ribosome and membrane preparations, only traces of labelled protein appear in the soluble fraction. There are indications that failure to observe accumulation of labelled protein in the soluble fraction (and possibly failure to obtain net synthesis of protein) is due to failure of the mechanism involved in 'splitting off' protein molecules manufactured on the RNP particles, so preventing passage into the soluble fraction and clearing of the protein-forming sites on the particulate fractions. However, study of certain preparations has resulted in limited synthesis of specific proteins in *in vitro* experiments.

Among mammalian systems intact reticulocytes form haemoglobin *in vitro* and it has been shown that ribosomes isolated from rabbit reticulocytes form labelled soluble haemoglobin when incubated in the presence of GTP, ATP, an ATP generating system, labelled amino acids and a pH 5 enzyme fraction, though the amounts formed are very small[4]. Similarly mammalian mitochondria bring about a net synthesis of cytochrome *c*. Not only are labelled amino acids incorporated into the protein, but incorporation occurs in positions expected from the known structure of cytochrome *c*[73]. Convincing evidence of synthesis of serum albumin by isolated microsomes of regenerating rat liver has been presented, although a net synthesis was not demonstrated[74]. Isolated microsomes of pea seedlings promote a net synthesis of soluble protein in the presence of appropriate supplements. The protein formed possesses enzymic activity, suggesting it is not a random chain of amino acids[33].

Turning to the study of subcellular preparations of bacteria, it has been shown that protoplasts or spheroplasts can synthesize a number of inducible enzymes (see p. 114), including arabinokinase in *B. subtilis*, β-galactosidase in *B. megaterium* and β-galactosidase, α-galactosidase, tryptophanase, ornithine transcarbamylase and aspartyl transcarbamylase in *E. coli*[55,75,76]. This is perhaps not surprising since it is known that osmotically fragile bodies produced from intact bacterial cells possess most of the biological attributes of the cells from which they are derived[55]. The subcellular preparations of *Staphylococcus aureus* obtained by ultrasonic treatment, when incubated under appropriate conditions, showed increases in the activity of several enzymes[77].

Several groups of workers also claim to have observed the synthesis of β-galactosidase in preparations derived from protoplasts or spheroplasts damaged by osmotic shock or lysed by digitonin[78-80]. One such preparation, consisting of protoplasts of *B. megaterium* subjected to carefully controlled osmotic shock, also displayed a considerable net increase in protein[78]. Net synthesis of protein has also been claimed for a particulate preparation derived from *Alcaligenes faecalis* as a result of ultrasonic treatment[67]. A preparation obtained from *B. subtilis* by lysis with lysozyme incorporated amino acids into protein and an increase in α-amylase activity was observed[62].

In most investigations with subcellular preparations of bacteria difficulties of interpretation exist due to the fact that the preparations are likely to

contain some intact cells, spheroplasts or protoplasts which could be responsible for the observed increases in enzyme activity. It is difficult to rule out the possibility that increases of enzyme activity are due to the metabolic removal of an inhibitor, the unmasking of already existing enzyme molecules or release of particle-bound enzyme. For example, in a study of ornithine transcarbamylase synthesis in lysed spheroplasts of *E. coli* it was shown that the increase in enzyme could be fully accounted for by synthesis in unbroken (lysis-resistant) spheroplasts present in the preparation and furthermore, synthesis in the rich milieu of the preparation occurred at a higher rate than in comparable numbers of spheroplasts in an environment less rich in nutrients[81]. In one investigation cited above[80] the preparation used contained a disturbingly high number of intact cells (about 2×10^6 viable cells/ml.). The system was remarkable in several other respects, which cannot be discussed in detail here, but which raise doubts about the validity of the claim that cell-free synthesis occurred and suggest that any enzyme and net protein synthesis occurring did so in the intact cells present in the preparations.

A particulate preparation obtained by breaking *E. coli* spheroplasts by the application of shearing forces displayed an increase in ornithine trans-carbamylase activity in the presence of appropriate supplements[81]. An indication that development of enzyme activity was not due to unruptured spheroplasts was obtained from the observation that increase in activity was ATP-dependent, this compound being without effect on intact cells or spheroplasts. Nevertheless, increase in ornithine transcarbamylase activity in disrupted spheroplasts represented only 1 to 2 per cent of the activity of an equivalent number of intact spheroplasts, so that the increase in activity could be due to release of particle-bound enzyme. Other experiments from the same laboratory provide some of the most convincing evidence for cell-free synthesis of specific enzymes in bacteria so far presented. A particulate preparation obtained from *E. coli* (strain B) displayed a twofold increase in β-galactosidase activity when suitably supplemented. Inclusion of ^{14}C-labelled leucine in the incubation mixtures resulted in the formation of labelled β-galactosidase, some of which appeared in the soluble fraction, the remainder being bound to the particles[82].

Many workers have noted the presence of various enzymic activities in washed ribosome preparations[4,45,83–85]. It is tempting to suggest that the activity is due to newly synthesized protein which has not yet left the site of synthesis. However, it has been pointed out[40] that proteins may become strongly adsorbed on to ribosomes and the enzymic activity ascribed to these particles may be due to contamination by soluble protein. Nevertheless several recent demonstrations of ribosome-bound enzymes appear not to be due to contamination of the preparations. Even after extensive washing the larger ribosomes of *E. coli* display β-galactosidase activity, that associated with ribosomes of induced cells being greater than the activity associated with ribosomes of non-induced cells[45,83].

In a strain of *Saccharomyces cerevisiae* β-glucosidase activity is associated with the 80 S ribosomes and cannot be completely removed by washing. Furthermore ribosomes of a strain incapable of forming β-glucosidase did not acquire activity when mixed with the soluble fraction from cells containing

constitutive β-glucosidase, nor did unlabelled ribosomes of the constitutive mutant retain radioactivity after mixing with ^{35}S-labelled soluble fraction, followed by extensive washing. Finally, short exposure of yeast cells to *p*-fluorophenylalanine, which prevents synthesis of active β-glucosidase, led to a virtual disappearance of ribosome-bound β-glucosidase activity, even though the soluble fraction of the cells contained large amounts of the enzyme. Ribosome-bound β-glucosidase activity reappeared within a few seconds of adding phenylalanine to reverse the effect of the analogue, suggesting that the β-glucosidase associated with the ribosomes is a precursor of the soluble enzyme[84]. These experiments demonstrate that ribosomes can be isolated without significant contamination by proteins present in the cytoplasmic soluble fraction. A study of ribosome-bound triosephosphate dehydrogenase activity in yeast and alkaline phosphatase activity in *E. coli* also led to the conclusion that the observed activity is not due to adsorption of enzymes from the soluble fraction[85].

As already noted, failure to observe net synthesis of most proteins *in vitro* may be due to failure of the newly completed molecules to vacate the site of formation, so blocking the site for further synthesis. Recent evidence suggests that this process, at least in pea seedlings, is enzymic and is dependent on the presence of ATP[33].

Incorporation of Amino Acid Analogues into Proteins

Amino acid analogues, that is substances produced by slight changes in the structures of natural amino acids by chemical substitution, are known to prevent the synthesis, in active form, of a number of enzymes in micro-organisms. Originally it was thought that analogues blocked synthesis of protein by competing with the natural amino acid during synthesis. In fact many analogues, rather than preventing protein synthesis, are incorporated into protein, at least some of the proteins formed under such conditions being biologically inactive. Many instances of incorporation of amino acid analogues into the proteins of animals and micro-organisms have been described[3,86] and only two aspects will be discussed, namely, incorporation into specific proteins and activation of analogues by amino acid activating enzymes.

Addition of certain analogues to growing cultures of *E. coli* and *Saccharomyces italicus* resulted in linear increase in cell mass, compared with an exponential increase in control cultures[16,87]. In *E. coli* cells grown in the presence of *p*-fluorophenylalanine or β-thienylalanine (both analogues of phenylalanine) 75 and 95 per cent of the phenylalanine of the cell proteins was replaced by the respective analogue. Similarly, growth in the presence of norleucine was accompanied by a reduction of methionine incorporation into protein[87–89]. Selenomethionine, in which the sulphur atom in methionine is replaced by selenium, replaced methionine for growth of a methionine-dependent strain of *E. coli*[90].

If the prior activation of amino acids is an obligatory step in the biosynthesis of protein, then analogues capable of incorporation into protein should be capable of activation. A purified preparation of pancreatic tryptophan activating enzyme activated 7-azatryptophan, tryptazan,

5- and 6-fluorotryptophan, but 5- and 6-methyl tryptophan were not activated and displayed, together with several other tryptophan analogues, the capacity to inhibit activation of tryptophan itself[91]. These results may be compared with incorporation studies which have shown that tryptazan and azatryptophan can be incorporated into proteins of *E. coli* and bacteriophage, whereas 4- and 5-methyl tryptophan, although leading to linear growth of *E. coli*, were not incorporated into protein[3,86,88]. 6-Methyl tryptophan, which failed to be activated by the tryptophan activating enzyme, also failed to inhibit growth of *E. coli*, nor was it incorporated into the protein of this organism[87,88,91]. The validity of comparing analogue activation by a pancreatic preparation with incorporation into *E. coli* proteins may be questioned. However *p*-fluorophenylalanine, β-thienylalanine, ethionine, selenomethionine and norleucine have all been shown to be activated by crude preparations of amino acid activating enzymes from *E. coli*[14,92]. Furthermore the incorporation of all these analogues into *E. coli* protein has been demonstrated[88–90].

Thus there appears a good correlation between ability of activating enzymes to activate certain analogues and the incorporation of analogues into protein. Amino acid analogues can also be bound to s-RNA[10,35], although this aspect of incorporation into protein requires further detailed study.

Many investigators have described impaired ability of micro-organisms to synthesize certain enzymes in active form in the presence of a wide variety of amino acid analogues[3,86]. For example the differential rate of synthesis (see p. 118) of β-galactosidase by *E. coli* was reduced in a methionine-dependent strain growing in the presence of selenomethionine[90]. Similarly the presence of *o*-, *m*- or *p*-fluorophenylalanine resulted in decreased activity of the enzyme alkaline phosphatase in *E. coli*[93]. However, from such investigations it is not possible to decide whether reduced enzymic activity is due to a reduction in the total amount of enzyme synthesized or to the production of normal amounts of enzyme molecules possessing a reduced specific enzymic activity compared with that of normal enzyme.

Recently the incorporation of analogues into highly purified specific proteins has been studied. Tritiated or ^{14}C-labelled *o*- and *p*-fluorophenylalanine have been shown to be incorporated into peptide linkages in several purified proteins of animal tissues[3,10]. Growth of *Bacillus cereus* in the presence of *p*-fluorophenylalanine or canavanine (an analogue of arginine) led to a decrease in the differential rate of synthesis of the enzyme penicillinase and the enzyme formed under these conditions behaved abnormally in immunological tests with antiserum induced by normal enzyme. Radioactive *p*-fluorophenylalanine was incorporated into cell proteins, including the immunologically abnormal penicillinase, the specific enzymic activity of which was lower than that of normal enzyme[94,95].

Ethionine replaced methionine in the α-amylase synthesized by a methionine-dependent strain of *Bacillus subtilis*, the resulting enzyme being indistinguishable with respect to its enzymic activity, electrophoretic mobility and some other physical characteristics. After growth in the presence of a mixture of methionine and ethionine 36 per cent of the

methionine content of the enzyme was replaced by ethionine[96]. Replacement of phenylalanine by *p*-fluorophenylalanine in the same enzyme amounted to 9 per cent. The electrophoretic mobility of the *p*-fluorophenylalanine-containing enzyme was identical with that of normal enzyme, although slight differences in certain other physical properties were detected. In contrast to the results obtained with ethionine, the enzymic activity of the *p*-fluorophenylalanine-containing enzyme was only about 70 per cent of that of the normal enzyme[97].

The flagella of bacteria may be removed by a variety of mechanical treatments and consist of a class of proteins known collectively as flagellins. Mechanical removal of the flagella of *Salmonella typhimurium* results in loss of motility, but not of viability and under appropriate conditions this organism can regenerate flagella. Since the flagella of this organism consists of a single protein, regeneration offers a suitable experimental system for the study of protein synthesis. The addition of *p*-fluorophenylalanine, β-2-thienylalanine or ethionine to cells growing in a suitable medium resulted in a decreased growth rate, although after incubation with β-2-thienylalanine or ethionine the cells possessed normal flagella and remained motile. After growth in the presence of *p*-fluorophenylalanine cells were non-motile but microscopic examination revealed the presence of abnormal 'curly' flagella, having a flagellar wave-length only half that of normal *Salmonella typhimurium* flagella[98]. Incorporation of *p*-fluorophenylalanine into the flagellin of this organism has not been demonstrated, although there is no doubt that the presence of this analogue results in formation of a biologically abnormal protein.

Non-flagellate forms may also be obtained by growing cells at 44°C and such cultures can regenerate flagella on transfer to medium at 37°C, although flagella are detectable only after a time lag. Although β-2-thienylalanine allowed synthesis of functional flagella in cells deflagellated mechanically, only non-functional flagella were produced in cells deflagellated by growth at 44°C. Evidence suggests that this may be due to failure to synthesize, in biologically active form, some component of the flagella-forming system[98].

Growth of bacteria in the presence of amino acid analogues does not result in the formation of detectable amounts of 'incomplete' proteins (peptides). Furthermore an analogue is incorporated equally into all the proteins of the cell, and there also appears to be an equal chance of an analogue replacing the corresponding natural amino acid at any site within a single protein molecule[89,96].

Thus there exists abundant evidence that analogues, closely resembling naturally-occurring amino acids in structure, can be incorporated into a variety of proteins in many different organisms. The proteins synthesized under these conditions may or may not retain their biological activity. It is tempting to suggest that substitution of an amino acid by an analogue probably results in impaired biological activity if one or more of the residues substituted occupy positions associated with the active catalytic site of the enzyme, whereas if the analogue is incorporated into other positions in the molecule then the resulting protein is likely to retain its biological activity. Changes in the physico-chemical behaviour of a protein may be brought about as a result of substitution at sites involved in the determination of secondary and tertiary structure.

ENVIRONMENTAL CONTROL OF ENZYME SYNTHESIS: ENZYME INDUCTION AND ENZYME REPRESSION

Although the ability of a micro-organism to produce a particular enzyme is genetically determined, the enzymic constitution of a microbial population is influenced to a large extent by physical and chemical factors in the environment. Furthermore the enzymic activity of micro-organisms varies widely with age of a culture, this phenomenon reflecting changes in constitution of the medium and changes within the cells. These effects, together with the influence of factors such as acidity of the medium, oxygen supply and temperature have been reviewed by Gale[99]. In the present account two effects, very different at first sight, though having much in common on closer examination, will be discussed, namely the synthesis and suppression of synthesis of specific enzymes under the influence of substances chemically or metabolically related to the substrate of the enzyme.

Induced Enzyme Synthesis

In 1900 Dienert studied the fermentation of galactose by yeast and noticed that the galactose was only fermented if galactose (or other closely related sugar) was incorporated into the growth medium. Cells grown on glucose could not ferment galactose, but did so if removed from the original growth medium and incubated for several hours with galactose under conditions where significant cell division did not occur. Glucose, on the other hand, was fermented immediately. This is one of many early investigations, frequently reviewed[99-102], which indicated that the synthesis of many microbial enzymes was influenced by exposure of the cells to the specific substrate of the enzyme. One such study led Karstrom to divide enzymes into 'adaptive', the formation of which was dependent on the presence of the specific substrate in the growth medium, and 'constitutive', the formation of which occurred irrespective of the nature of the growth medium. Later the distinction between 'adaptive' and 'constitutive' enzymes was thought to be less rigid than the original definition would suggest since some enzymes are produced constitutively in considerable amounts; production is stimulated to higher levels by the presence of the substrate. In addition most, if not all, adaptive enzymes are formed in small amounts in non-adapted cells. Nevertheless there is a real distinction between adaptive and constitutive enzymes since bacterial strains possessing an enzyme which is typically adaptive can, by spontaneous mutation, give rise to constitutive strains.

The earlier investigators of adaptive phenomena all interpreted their observations in terms of synthesis of specific enzymes in response to the presence of the enzyme substrate, though in many instances alternative explanations were possible including, in those experiments involving growth in the presence of the substrate, spontaneous mutation and selection of mutants possessing the particular enzymic activity studied[100]. However considerable circumstantial evidence suggests that synthesis of some protein component is involved in the development of adaptive enzyme activity, but not until recently was the first conclusive evidence of adaptive synthesis of a

specific protein species forthcoming. Rabbit antiserum was prepared against a purified preparation of β-galactosidase from *E. coli* adapted to lactose (a β-galactoside). This antiserum precipitated the enzyme without loss of enzymic activity (measured by ability to hydrolyse β-galactosides) and a comparison between extracts of glucose-grown cells and lactose-grown cells revealed that adaptation to lactose resulted in an increase in a specific antigenic component, the amount corresponding quantitatively to the increase in enzymic activity measured by a suitable assay method[103]. Usually in investigations of adaptive enzymes the *activity* of the enzyme has been measured and the assumption made that the value obtained is a valid estimate of the *amount* of enzyme present in the cells.

The term 'adaptation' has frequently been used in describing microbiological phenomena, which, although often having the same over-all effect, are very different mechanistically. To overcome such ambiguities it was proposed, in 1953, that the term 'induced enzyme formation' should replace 'adaptive enzyme formation'. The phenomenon may be defined as the synthesis of a specific enzyme protein under the influence of a substance (the inducer) which may or may not be a substrate of the enzyme. The synthesis of induced enzyme is a phenotypic change occurring against the background of a fixed genotype[103]. Behaviour of an *E. coli* culture induced to form β-galactosidase may be used to illustrate the characteristics of synthesis of an induced enzyme. In a culture of *E. coli* growing in a medium containing succinate as sole carbon source the β-galactosidase activity of the cells is very small, but if a suitable inducer is added there is an increase of about 1,000-fold in the rate of synthesis of the enzyme. Removal of the inducer by centrifuging and resuspending the cells in fresh medium (without inducer) results in rapid cessation of further enzyme synthesis[103].

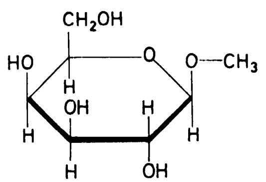
Basal enzyme

Providing a sufficiently sensitive assay is available it is possible to demonstrate low 'basal' levels of inducible enzymes in non-induced cells. Available evidence suggests that the 'basal' enzyme is identical with the enzyme synthesized on the addition of inducer. For instance, preparations of 'basal' β-galactosidase of *E. coli* and *Neurospora crassa* and penicillinase of *Bacillus cereus* and *Bacillus subtilis* are immunologically and physico-chemically indistinguishable from the respective induced enzymes obtained from the same strains[100,102]. Tests of this type allow the important conclusion that the addition of an inducer promotes a quantitative increase in an enzyme already produced in minute amounts in the absence of the external stimulus. The inducer does not introduce information required for the synthesis of the specific enzyme; the information already exists in the non-induced cells, the inducer serving to 'trigger off' an increased rate of synthesis.

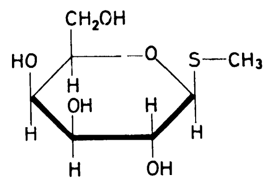
Specificity of induction

Early investigations indicated that enzyme induction is a highly specific process, but it soon became apparent that enzymes may be induced by substances other than the natural substrate (or substrates) of the enzyme. However, such inducers are always structurally closely related to the natural substrate[100].

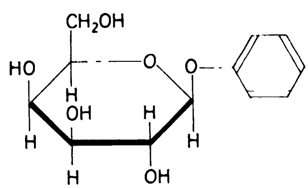
In 1951 Monod and his colleagues, using *E. coli*, strain ML, examined the ability of a number of β-galactosides to act as substrates and inducers of β-galactosidase. The information has been extended more recently to β-thiogalactosides[102,104]. The investigations revealed that inducers of the enzyme need not be substrates of the enzyme, nor substrates inducers, that is, the role of substrate and inducer are distinct. This difference was well illustrated by a simple experiment in which some of these compounds were incorporated into the growth medium as sole sources of carbon[104]. Methyl-β-D-thiogalactoside failed to support growth since, although capable of



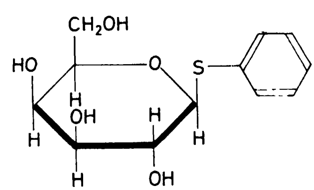
Methyl-β-D-galactoside
SUBSTRATE + INDUCER



Methyl-β-D-thiogalactoside
INDUCER (not substrate)



Phenyl-β-D-galactoside
SUBSTRATE (not inducer)



Phenyl-β-D-thiogalactoside
Neither substrate nor inducer

Figure 3.3

acting as an inducer, it was incapable of utilization by the enzyme. Similarly phenyl-β-D-galactoside failed to support growth, but for a different reason. This compound, although a competent substrate, was unable to induce the formation of the enzyme. If, however, the two compounds were added together growth resulted, the β-thiogalactoside induced the enzyme responsible for the utilization of the phenyl-β-D-galactoside as a source of carbon.

The β-thiogalactosides are not hydrolysed by β-galactosidase, nor can they be used as a carbon source for growth, yet many are excellent inducers of the enzyme, a property which renders them useful in the study of the kinetics of enzyme synthesis. Phenyl-β-D-thiogalactoside does not induce β-galactosidase, nor is it a substrate of the enzyme, but if added to growing cells in the presence of the inducer methyl-β-D-thiogalactoside induction is inhibited. The structure of some of these β-galactosides and β-thiogalactosides, together with an indication of their ability to induce and act as substrates of *E. coli* β-galactosidase is shown in *Figure 3.3*.

The ability of a comparable series of β-galactosides and β-thiogalactosides to act as inducers and substrates of β-galactosidase in *Bacillus megaterium* has been tested, with similar results, namely that ability to induce is independent of ability to act as substrate[100]. However some compounds which were good inducers of the *B. megaterium* enzyme possessed little or no activity as inducers of the *E. coli* enzyme. Similarly β-glucosides and β-thioglucosides vary widely in their capacity to induce and act as substrates of β-glucosidase in *Saccharomyces cerevisiae*[105]. Of a number of different penicillins and penicillin-like compounds one, cephalosporin C, induced the formation of penicillinase in *B. cereus* but was not a substrate of the enzyme[100].

Pseudomonas fluorescens forms an inducible mandelic acid racemase which leads to the formation of a racemic (DL) mixture of mandelate in the presence of either L- or D-mandelate as substrate. This enzyme can be induced by either optical isomer[106]. Similarly the inducible methionine racemase of a closely related organism converts either L- or D-methionine to a racemic mixture. In this instance only the D-isomer is a competent inducer[107]. The tyrosinase of *Neurospora crassa* attacks the L- and D-isomers of a variety of aromatic amino acids. The enzyme is induced by all these compounds, although the D-isomers are much better inducers than the corresponding L-isomers[108]. However, all these enzymes are active against both L- and D-isomers, but recently instances of induction of an enzyme by optical isomers which are not substrates for the enzymes have been revealed. The histidase of *Paracolobactrum aerogenoides*, which deaminates L-histidine to urocanic acid, is induced by D-histidine though this isomer is not a substrate. Urocanic acid is the substrate of another inducible enzyme, urocanase, which is also induced by D-histidine[109]. In unpublished work Bocks and Tristram have found a similar example of induction by a D-isomer of an enzyme active only on the corresponding L-isomer in *Lactobacillus arabinosus*, in which the malic enzyme is induced by both L- and D-malate, but decarboxylates only L-malate.

As a result of many investigations of the specificity of induction of a wide variety of enzymes[100,102,105] it appears that, although an inducer need not be a substrate of the enzyme, it must have certain chemical features in common with the substrate.

Stanier[101] has studied a number of bacterial metabolic pathways, including the degradation of aromatic compounds, involving a sequence of inducible enzymes. Addition of a single compound, the substrate of the first enzyme in the sequence, induces the formation of this enzyme and the product of the enzyme action induces, and is also the substrate of, the second enzyme, and so on. Such a phenomenon is known as 'sequential induction', the over-all result being the synthesis of a number of enzymes as a result of the addition of a single compound. The metabolism of galactose by yeast involves three inducible enzymes acting sequentially. However, in at least one strain, sequential induction of the type described by Stanier does not appear to be involved, since galactose itself apparently induces all three enzymes independently[105].

A number of enzymes involved in aerobic respiration of micro-organisms are induced by transfer from anaerobic to aerobic conditions. For instance, enzymes responsible for operation of the Krebs cycle are absent in

anaerobically grown *Pasteurella pestis* but are rapidly synthesized on aeration of suspensions of such cells[110]. It is probable that these enzymes are not directly induced by oxygen, but the precise mechanism of induction has not been investigated. Similarly anaerobically-grown yeast lacks cytochrome *c*, but formation of this protein is induced by oxygen[111].

Kinetics of induced enzyme synthesis

As pointed out by Pollock[100] the type of curve obtained when increase in induced enzyme activity is plotted against time differs greatly, depending particularly on the nature of the inducer and whether growing or 'non-growing'* cells are studied. Suspensions of yeast cells removed from the growth medium and suspended in suitable buffer to which galactose was added formed galactozymase slowly at first, but the rate gradually increased. Ultimately increase in enzyme activity ceased, resulting in a sigmoid curve when plotted against time. A similar relationship has been demonstrated in a variety of other inducible systems in both yeasts and bacteria. This type of kinetics probably reflects the fact that the inducer, which is also a substrate, is itself providing energy, or in some instances, carbon or nitrogenous material (or a combination of these) for the synthesis of enzyme. In the early stages of enzyme formation the small amount of enzyme present is limiting its own synthesis[100]. Furthermore, the concentration of an inducer which is also a substrate changes constantly during the experimental period.

Other systems studied in washed 'non-growing' cells do not display the period of acceleration observed in the instances described above. Enzyme synthesis commences from the time of addition of inducer and proceeds at a constant rate until it finally stops, more or less abruptly. Such kinetics have been described for the synthesis of α-amylase induced by maltose or starch in *Pseudomonas saccharophila*[100], D-histidine-induced synthesis of histidase in *Paracolobactrum aerogenoides*[109] and in other systems[100].

The discovery of inducers not detectably metabolized by the enzyme induced, nor utilized as a source of carbon or metabolic energy by the organism investigated, presented opportunities for measuring induced enzyme synthesis under conditions where the inducer concentration does not change measurably during the experiment, and where the activity of the enzyme synthesized is never a limiting factor in its own synthesis. This procedure obviates non-specific effects observed when, as a result of the metabolism of the inducer, carbon, nitrogen or energy are made available. Such conditions have been termed 'gratuitous'[102] and can most readily be achieved by adding a 'non-metabolizable' inducer to an exponentially growing culture. The use of exponentially growing cultures represents the simplest approach to the attainment of physiological steady-state conditions in a bacterial culture, and allows a comparison to be made between the rates of synthesis of total cell protein and a specific protein (induced enzyme). The addition of sufficient β-thiogalactoside inducer to saturate the induction mechanism to an *E. coli* culture containing succinate as carbon and energy source results in formation of β-galactosidase. If the amount of enzyme produced under these conditions is plotted against the increase in total cell

* 'Non-growing' cells in this context refers to the absence of cell division or net increase in dry weight.

protein, a linear relationship is obtained. In effect this means that the induced enzyme produced represents a constant fraction of the total protein synthesized. The slope of the line represents that proportion of new cell protein which is induced enzyme and has been termed the 'differential rate of synthesis'. The differential rate of synthesis is constant, although different β-thiogalactosides lead to different differential rates of synthesis (*Figure 3.4*) *i.e.* even among those β-thiogalactosides which are competent inducers, not

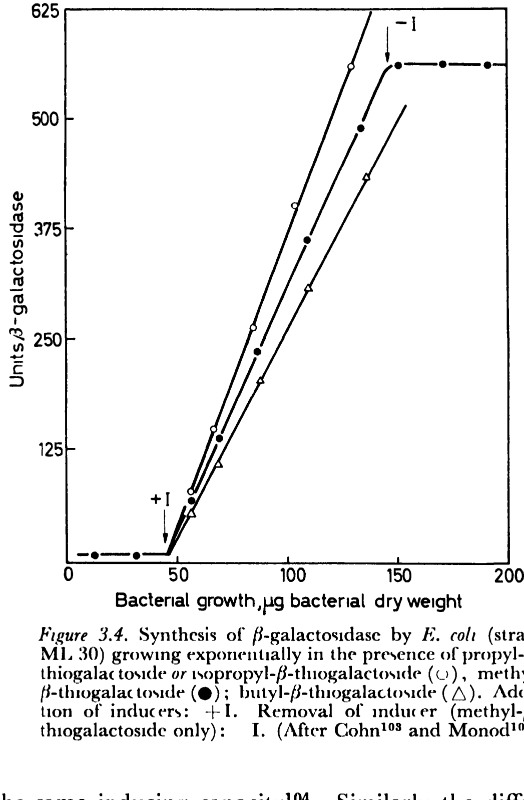


Figure 3.4. Synthesis of β-galactosidase by *E. coli* (strain ML 30) growing exponentially in the presence of propyl-β-thiogalactoside *or* isopropyl-β-thiogalactoside (○), methyl-β-thiogalactoside (●); butyl-β-thiogalactoside (△). Addition of inducers: +I. Removal of inducer (methyl-β-thiogalactoside only): I. (After Cohn[103] and Monod[101])

all have the same inducing capacity[104]. Similarly the differential rate of synthesis of yeast β-glucosidase also varies with different inducing β-thioglucosides[105]. Under 'conditions of gratuity' a constant differential rate of synthesis of malic enzyme induced by L- or D-malate in *Lactobacillus arabinosus* and of a number of other enzymes[105] has been observed.

In earlier investigations synthesis of most induced enzymes, including β-galactosidase, appeared to commence without delay after addition of the inducer (*Figure 3.4*). However, a recent re-investigation of induced β-galactosidase, tryptophanase and D-serine deaminase synthesis in *E. coli* demonstrated a lag of about 3 minutes (at 37°C) between addition of inducer and appearance of enzyme activity[112]. The significance of this short

lag period, which was prolonged at lower temperatures, will be discussed later.

In practically all systems examined[103,112] removal of the inducer following a period of induced enzyme formation results, within a few minutes, in cessation of further enzyme synthesis (*Figure 3.4*). Unlike most other induced enzymes the synthesis of the (largely extracellular) inducible penicillinase of *B. cereus* continues after removal of the inducer[100]. The increase in amount

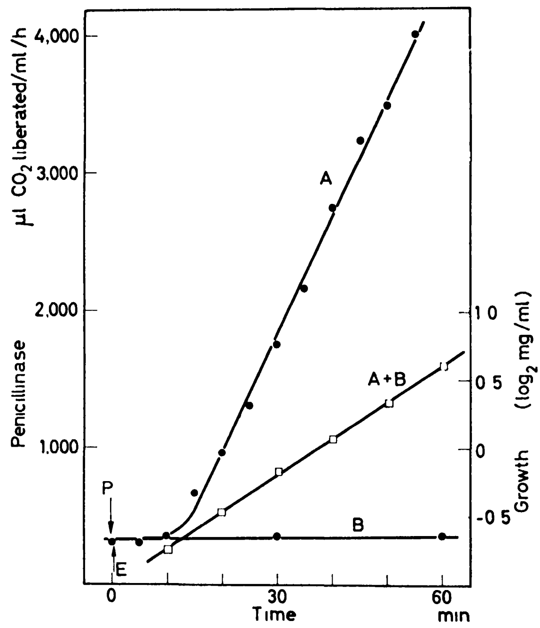


Figure 3.5. Formation of penicillinase in exponentially growing cultures of *Bacillus cereus*[104]
A, After transitory exposure to penicillin (1 unit/ml), B, Control, without penicillin, P, Penicillin added to A; E, Penicillinase added to A and B to destroy free penicillin; □ growth; ● penicillinase formed.

of penicillinase after transitory (1 minute) exposure of exponentially growing cells to penicillin is linear with time, but is preceded by a lag of about 15 minutes before synthesis begins (*Figure 3.5*). Although free penicillin in the medium may be removed by washing or by destruction with added penicillinase, the addition of ^{35}S-labelled penicillin to *B. cereus* results in the retention of some radioactivity in the cells. This 'bound' radioactivity, which corresponds to some 100 ^{35}S atoms/cell, cannot be removed by washing, nor is it lost during subsequent growth. That the inducer taken up by the cells does not exist as free penicillin within the cells is almost certain since it would be destroyed by the penicillinase formed. Although the precise significance of the 15-minute lag observed between addition of penicillin and

the appearance of penicillinase is not understood it is known that: (*a*) the lag involves the metabolism of the cells since it is prolonged by periods of anaerobiosis or by treatment of cells with ultra-violet light, and (*b*) since the synthesis of a penicillinase molecule occupies only about 2 minutes the lag is not a reflection of the time required for synthesis of the first enzyme molecules. It has been suggested[100] that the inducer combines with some receptor site in the cell to form an inducer–receptor complex. On this basis the difference between induction of penicillinase and induction of other enzymes, in which synthesis stops on removal of inducer, would be that the complex in the penicillinase induction system is metabolically stable, whereas in other systems the complex is unstable, its presence being assured only by the continued presence of inducer.

Precursors of induced enzymes

Non-induced cells of a variety of bacterial species capable of forming inducible β-galactosidase contain a protein (Pz) which cross-reacts with anti-β-galactosidase serum. Preliminary investigations, including the strong positive correlation between ability to form Pz and inducible β-galactosidase suggested that the former might be a complex precursor of the latter, being converted to active β-galactosidase by comparatively minor changes provoked by the presence of an inducer. This aspect of induced enzyme formation cannot be examined in detail, but as a result of labelling experiments with *E. coli* it was shown that appreciable amounts of a complex enzyme precursor do not exist in non-induced cells and that Pz, which exists in appreciable amounts in such cells cannot be the precursor of β-galactosidase which, in growing cells, is synthesized *de novo* from amino acids formed in cells after addition of inducer[103]. This conclusion has recently been confirmed by experiments which show that ability to synthesize β-galactosidase is not invariably accompanied by ability to synthesize Pz[113]. *Shigella dysenteriae* lacks the ability to produce either Pz or β-galactosidase. Genetic material from *E. coli* can be introduced into *Shigella* either by conjugation (see p. 124) or by bacteriophage-mediated genetic transfer (transduction). By these means the ability to synthesize β-galactosidase, immunologically indistinguishable from the *E. coli* enzyme, was transferred to *Shigella dysenteriae*, but in none of these recombinants could Pz be detected[113].

An intensive study of the inducible penicillinase of *Bacillus cereus* also led to the conclusion that no appreciable quantities of complex (protein-like) precursor exist in cells before induction, but that the enzyme is formed *de novo* from its constituent amino acids after addition of inducer[100].

Inducible transport mechanisms

Many examples have been described of the failure of intact microbial cells to metabolize particular substrates, although extracts of the same strains possess the necessary enzyme (or enzymes). For example, intact cells of a strain of *E. coli* failed to utilize exogenous glucose, though maltose was metabolized, giving rise to intracellular glucose which was degraded by the cells. This suggests that the cells possessed enzymes responsible for glucose degradation, but that intact cells could not utilize externally added glucose. Many similar situations have been uncovered[114]. Although not the only

possible explanation, such observations were usually ascribed to the impermeability of the cells to the substrate concerned, and the results with maltose and glucose utilization in *E. coli* infer that penetration is highly selective since the *E. coli* strain studied appeared to be permeable to the former, but impermeable to the latter. Failure in this way to metabolize a substrate, notwithstanding the presence of the necessary enzymes in the cell has been referred to as 'crypticity', or the 'cryptic state'.

Intact cells of *Pseudomonas fluorescens* grown on fumarate or succinate fail to oxidize citrate, isocitrate or α-ketoglutarate immediately, but do so after a period of exposure to these substrates. This adaptive response to Krebs cycle intermediates is abolished by treatments known to prevent induced synthesis of active enzymes (exposure to ultra-violet light or the amino acid analogues ethionine and *p*-fluorophenylalanine). However, extracts of the succinate- or fumarate-grown cells contain the enzymes necessary for the operation of the Krebs cycle and it has been suggested that during the period of adaptation before rapid oxidation of the intermediates occurs, synthesis of some protein component necessary for penetration of the substrates into the cell is effected. A similar situation has been described in acetate-grown cells of *Pseudomonas aeruginosa* and in an *Aerobacter aerogenes* mutant[114].

An inducible system, responsible for the passage into, and accumulation of thiogalactosides within *E. coli* cells has been described in detail[114,115].

Cells induced by growth in the presence of a β-galactoside show an energy-dependent reversible accumulation of large quantities of ^{35}S-labelled β-thiogalactosides. Non-induced cells show no such activity. The material accumulated by induced cells may be quantitatively recovered by extraction with boiling water, by far the greater part being present as unaltered thiogalactoside, although recent investigation has detected the acetylation of about 5 per cent of the thiogalactoside accumulated. Extracts of cells lacking ability to accumulate thiogalactosides also fail to acetylate these compounds, suggesting an obligatory connection between the two processes[116]. This system has been termed 'galactoside permease' since it possesses several properties consistent with an enzymic nature. For instance, the system is highly specific for galactosides; it is induced specifically by a variety of α- and β-galactosides and thiogalactosides, and the induction is effective only under conditions which allow protein synthesis[114,116]. A comparison of β-galactosidase synthesis in a wild-type *E. coli* strain possessing inducible 'galactoside permease' and in a 'cryptic' strain (lacking the ability to concentrate β-thiogalactosides in the cell) has led to an explanation of the anomalous kinetics of β-galactosidase formation in wild-type strains exposed to low concentrations of inducers[104,105,114].

A considerable number of bacterial and yeast 'permeases' have now been described, some inducible, others constitutive[105,114].

Repression of Enzyme Synthesis

Bacteria capable of growth in simple defined media containing inorganic salts and a suitable organic carbon source are able to synthesize all the complex compounds required for growth. Since these compounds are formed at different rates it might be expected that some of them would be

formed in excess of growth requirements. Generally speaking, large excesses of the precursors of proteins (amino acids) and nucleic acids (purines and pyrimidines) and intermediates in the formation of these and other cell constituents do not accumulate in the growth medium. Furthermore it has frequently been demonstrated that if an amino acid, purine or pyrimidine is supplied to such an organism, then synthesis of the particular metabolite ceases. A consideration of these facts leads to the conclusion that the growing cell possesses mechanisms for the control of synthesis of intermediates concerned in biosynthesis and that these controls must be highly integrated[117].

The production of an intermediate could clearly be controlled in two ways, firstly by inhibition of the *action* of an enzyme responsible for synthesis of the compound, and secondly by inhibition of *synthesis* of the enzyme itself. Examples of both types have been described and appear to constitute important mechanisms for the control of biosynthesis in bacteria. The first type of control (inhibition of enzyme *action*) does not directly concern us in a discussion of protein synthesis and will not be discussed further. The second type of control (inhibition of enzyme synthesis) was first demonstrated during studies on the synthesis of tryptophan and methionine synthetases in bacteria. Incorporation of tryptophan or methionine (the respective products of these two enzymes) into suitable media resulted in failure of the organisms to produce the relevant enzyme[117]. Methionine, when added to growing cells of *E. coli* already containing the enzymes for methionine synthesis, prevented further synthesis of methionine synthetase, but there was no loss of existing enzyme, although the enzyme content per cell decreased as a result of 'diluting out' of existing enzyme[118].

In the presence of exogenous arginine, synthesis of this amino acid ceases in *E. coli* and under these conditions the cells contain only low levels of the enzyme acetylornithinase, one of the enzymes involved in arginine formation. Synthesis of the enzyme is also prevented by the presence of ornithine, an intermediate in the synthesis of arginine. One mechanism of control of enzyme synthesis by low molecular weight compounds (enzyme induction) was already well known and Vogel coined the term 'enzyme repression' for the analogous phenomenon of reduction of rate of enzyme synthesis by a low molecular weight compound. Although having the opposite effect, both mechanisms allow the cell to control, within the rigid framework of a fixed, genetically-determined potentiality for enzyme synthesis, the enzymic constitution most consistent with metabolic efficiency under particular environmental conditions. The presence of arginine in the growth medium also represses the formation of three more enzymes, acetylornithine-δ-transaminase, ornithine transcarbamylase and arginosuccinase, all implicated in the biosynthesis of arginine[119,120]. In addition the formation of a transport mechanism responsible for entry of its precursor, acetylornithine, into cells of *E. coli*, is repressed by arginine[121].

Even the (relatively) low concentrations of free arginine present in wild-type *E. coli* growing in the absence of exogenous arginine are sufficient to partially repress ornithine transcarbamylase synthesis, the enzyme being produced in considerably larger amounts if the internal free arginine concentration is reduced by experimental manipulation[119,122]. The degree of repression is only moderate if the arginine supply in the medium does not exceed that

which can be utilized for protein synthesis. Concentrations in excess of this level lead to marked repression[123]. Similar effects involving formation of higher enzyme levels when the internal levels of a metabolite are reduced experimentally have been noted with enzymes concerned in pyrimidine and tryptophan biosynthesis[124,125].

Five of the enzymes concerned in biosynthesis of histidine in *Salmonella typhimurium* are repressed by the presence of histidine in the growth medium[126,127]. By growing histidine-dependent mutants in the presence of formylhistidine or histidinol, both of which limit the amount of histidine available to the organism, it was shown that states of partial repression could readily be obtained and that the level of each enzyme tested was affected *to the same extent*. This phenomenon was termed 'co-ordinate repression'[126]. To what extent co-ordinate repression may be a general phenomenon is not known, but three enzymes concerned in pyrimidine biosynthesis, although repressible, are not co-ordinately repressed[124]. Of considerable interest is the observation that histidine, while repressing the enzymes of histidine biosynthesis to the same extent, also strongly inhibited the action of the first enzyme in the sequence[127], but did not repress the formation of the histidine activating enzyme[128]. Consequently the presence of histidine in the growth medium prevents the synthesis of histidine, but does not prevent its incorporation into protein.

Although enzyme induction is provoked by the enzyme substrate (or closely related compound), repression of enzyme synthesis is effected by the product of enzyme action (or by analogues of the product), or, in those instances involving a sequence of enzymes, by the final product of the sequence. That the repression of all enzymes of the arginine and histidine biosynthetic sequences are repressed by the final products of the sequence (arginine and histidine respectively) and not by the product of each individual enzyme as a result of reversibility of enzyme action is established by the fact that mutants blocked at some intermediary point in the sequence suffer repression of enzymes previous to the genetic block. Furthermore, although arginine or ornithine repress ornithine transcarbamylase in wild-type *E. coli*, neither ornithine nor any other intermediate represses this enzyme in mutants which are unable to convert the intermediate to arginine.

An interesting example of repression is seen in the production of isocitratase in bacteria grown on acetate as sole source of carbon. This enzyme, which splits isocitrate to succinate and glyoxylate, is a key enzyme in the supply of four-carbon intermediates from acetate by the glyoxylate cycle. It is formed in large amounts in *Pseudomonas ovalis* (Chester) or *Micrococcus denitrificans* growing on acetate, but its production is considerably decreased by growth on succinate or certain other tricarboxylic acid cycle intermediates[129]. *M. denitrificans* can also grow autotrophically on carbon dioxide as sole carbon source and under these conditions the cells contain no detectable isocitratase. The presence of succinate in the growth medium also represses the formation of isocitratase in *E. coli*[130].

Repression of enzyme synthesis in micro-organisms appears to be a phenomenon of widespread occurrence. The many examples now studied will not be described in detail, but include, besides those already mentioned,

enzymes concerned in the synthesis of pyrimidines, purines, proline, isoleucine, valine and bacterial photosynthetic pigments[117,119,124,131]. The synthesis of alkaline phosphatase, which splits inorganic phosphate from a wide variety of phosphorylated organic compounds, is repressed by the presence of inorganic phosphate in the growth medium in some strains of *E. coli*[132,133] and in *B. subtilis*[134].

Genetic Regulation of Enzyme Synthesis: The Relationship Between Enzyme Induction and Repression

There now exists abundant evidence, which will be discussed below, that the primary structure, or amino acid sequence, of proteins is under genetic control. Recent investigation of the synthesis of some enzymes in bacteria has revealed genetic control of the phenotypic expression of genes determining protein structure, so controlling the amount of individual proteins found in cells.

E. coli, the organism in which an intensive study of the synthesis of several enzymes has been made, is also one of the few bacteria in which genetic analysis is possible. One type of genetic transfer in this organism occurs as a result of conjugation between a cell of a donor strain (F^+ or *Hfr*) and a cell of a recipient strain (F^-). The transfer, which is unidirectional, occurs as a result of the injection of genetic material from donor to recipient; it may be interrupted by mechanical separation of the participants in conjugation. In this way, by scoring the recombinants from such interrupted matings it has been determined that the genetic characters are linearly arranged along a single linkage group and always penetrate into the recipient cell in a predetermined order, commencing from the same extremity. Interruption of mating still allows the incorporation of the genetic material already transferred into the genome of the recombinant cell, but prevents further transfer[135]. During conjugation transfer of cytoplasmic material does not occur.

Normal (wild-type) strains of *E. coli* possess the ability to synthesize the enzyme β-galactosidase responsible for hydrolysis of β-galactosides, including lactose. The synthesis of the enzyme is inducible in wild-type strains, which also possess an inducible transport mechanism (galactoside permease) responsible for the passage into and accumulation of galactosides within the cell. The capacity to synthesize β-galactosidase, the galactoside permease and the control of synthesis of both these components has been shown to be determined by three genes, z, y and i respectively. Wild-type strains are designated $z^+ y^+ i^+$.

Many mutant strains (Lac^-), having impaired ability to utilize lactose have been isolated. These Lac^- mutants fail to grow on lactose for differing reasons and fall into well-defined physiological groups, the most important being, for the purpose of this discussion:

(*a*) Mutants (designated z^-) which fail to form active β-galactosidase under any conditions, but several synthesize an antigenically related, though enzymically inactive, protein[136]. This, and other available evidence, suggests that the z gene determines the structure of β-galactosidase.

(*b*) Mutants (designated y^-) which fail to synthesize galactoside permease, but possess normal levels of β-galactoside—the so-called 'cryptic' mutants[114].

In addition, other mutants (designated i^-), in which both β-galactosidase and galactoside permease are produced without the intervention of exogenous inducers, *i.e.* constitutively, have been obtained. The β-galactosidase formed by i^+ (inducible) and i^- (constitutive) strains are identical immunologically and in their relative affinities for various β-galactosides[103]. All three genes are closely linked in the *Lac* region of *E. coli* K-12 chromosome, though all are independent[137].

The synthesis of β-galactosidase in zygotes arising from crosses between the mutant types enumerated above has been investigated[137], some of the experiments being detailed in *Table 3.2.* The main conclusions drawn from these observations are: (*a*) The phenotypic expression of the z^+ allele is

Table 3.2. Synthesis of β-galactosidase in zygotes derived from conjugation of *E. coli* strains[137]. (For terminology and details see text)

Experiment	*Hfr* (donor)	F^- (recipient)	*Inducer* *	β*-Galactosidase synthesis*
I	z^+i^+	z^-i^+	+	Synthesis commenced within a few minutes of transfer of z^+ to zygotes
II	z^-i^-	z^+i^+	—	*No* enzyme synthesis
III	z^+i^+	z^-i^-	—	Enzyme synthesized *for limited period* then ceased unless inducer added

* 10^{-3} M isopropyl-β-D-thiogalactoside

very rapid (Experiment I). (*b*) In Experiments II and III (which represent reciprocal crosses) neither parent is able to synthesize β-galactosidase since in one parent (z^+i^+) enzyme synthesis requires the presence of an inducer and the other (z^-i^-) lacks the ability to produce active enzyme. The results of Experiment II suggests that i^+ (inducibility) is dominant over i^- (constitutivity) since the zygotes contain entirely F^- (i^+) cytoplasm and although they receive the i^- allele from the donor this allele is never expressed and β-galactosidase is not synthesized in the absence of inducer, *i.e.* they behave as inducible cells. The results of Experiment III confirm this conclusion since, although enzyme synthesis proceeds for 1 to 1·5 hours in the absence of inducer (constitutive synthesis), the i^+ (inducible) allele acquired from the donor parent is expressed and the cells cease formation of enzyme *unless inducer is added*, i.e. the zygotes, originally constitutive, become inducible.

Of several possible models examined to account for these results the most likely is the supposition that, in inducible strains the synthesis of the enzyme and permease is inhibited by a repressor substance formed under the influence of the i^+ allele. The exogenous inducer antagonizes this repressor and in the presence of the i^- allele active repressor is not synthesized and the requirement for exogenous inducer disappears (constitutive behaviour).

The picture emerging from these studies on *Lac*$^-$ mutants of *E. coli* is one in which the activity of the β-galactosidase system (β-galactosidase plus galactoside permease) is controlled by at least three genes, one (z) controlling the structure of the enzyme itself, another (y) controlling the structure of the permease and the third (i) controlling the *phenotypic expression of the z and y genes.* The importance of these observations lies particularly in the fact that they present the phenomenon of enzyme induction as a specific antagonism of a repressor substance, synthesized under the control of the i gene.

In *E. coli* the synthesis of the sequence of enzymes responsible for tryptophan formation is repressed by the addition of tryptophan to the medium. 'Non-repressible' mutants have been isolated, in which the repressor effect of tryptophan is abolished for all enzymes of the sequence. These mutations affect a gene, distinct from those determining the capacity to synthesize the individual enzymes, the function of which appears to be to cause the synthesis, in the presence of tryptophan, of a repressor which prevents the phenotypic expression of the genes responsible for formation of each enzyme in the biosynthetic sequence[12].

These and related observations have led to the generalized hypothesis that the control of enzyme synthesis is under the influence of a 'regulator'

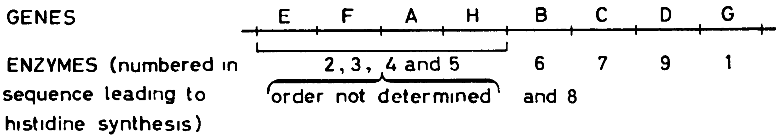


Figure 3.6

gene (termed *i* in the β-galactosidase system) distinct from the genes determining the *structure* (*i.e.* amino acid sequence) of enzymes. Regulator genes are thought to control the function, and hence phenotypic expression of genes of 'structure' by action on a site in the genome, termed the 'operator'. The operator is visualized as being adjacent to a group of structural genes all concerned with the same biosynthetic sequence. Under the influence of repressor produced by the regulator gene the expression of 'structural' genes relating to a single biosynthetic sequence can be inhibited through the operator[138]. The whole group of genes, together with the operator controlling their expression, has been termed an 'operon'.

Mutations leading to constitutive production of an enzyme may then be regarded as a change in the operator resulting in loss of sensitivity for the product of the corresponding regulator gene, or as a change in the regulator gene leading to failure to synthesize an active repressor molecule[138]. Evidence supporting this hypothesis has been obtained from a study of the *Lac* segment of the *E. coli* chromosome. The operator gene (*o*) appears to be distinct from the *i* gene, though linked to the other genes of the *Lac* segment, the order being *y*, *z*, *o*, *i*. Two distinguishable types of constitutive mutants have been isolated—the i^- mutants already mentioned and mutations of the operator gene (o^c) which also lead to constitutive production of both β-galactosidase and galactoside permease. Other point mutations (o^0) behave like genetic deletions, in that these mutants form neither β-galactosidase nor the permease under any conditions, due presumably to complete loss of function of the *o* gene[138].

Further evidence in support of this hypothesis comes from a study of the enzymes responsible for histidine biosynthesis in *Salmonella typhimurium*[127]. This system involves nine enzymes controlled by eight 'structural' genes linked in a 'cluster' as shown in *Figure 3.6.* It has already been mentioned that the formation of five of these enzymes (the remainder have not yet been studied in detail) is co-ordinately repressed by histidine. Study of three histidine-dependent mutants revealed the existence of a small region of the

chromosome which controls all the genes concerned with the synthesis of the histidine biosynthetic enzymes. These mutants have in common the deletion of a portion of the chromosome in the C, D and G gene regions, but lack *all* the histidine biosynthetic enzymes, notwithstanding the fact that most of the 'histidine' genes are located outside the mutated region. It has been postulated[127] that the histidine 'cluster' of genes constitutes an 'operon' controlled by an operator, non-functional as a result of the deletions in the three mutants described and located near the end of the gene 'cluster', though the precise location remains undetermined.

These investigations leave unanswered the question of the chemical nature of the repressor, although it is probably not protein and it has been suggested that it is nucleic acid[139]. It would appear that inducers and repressor metabolites do not act at the site of protein synthesis, as originally proposed in many attempts to explain their mode of action, but rather that they control, in an indirect way not yet understood, the expression of the 'structural' genes, *i.e.* the control is at the gene level and not at the enzyme forming site. This could occur as a result of complex formation between inducer, or repressor metabolites (or a product of their prior metabolism) with the product of the regulator gene (nucleic acid?) and subsequent combination of the complex with the operator.

Regulation of the synthesis of tyrosinase in *Neurospora* is under the control of two genes which are not closely linked and which are distinct from the gene determining the structure of the enzyme. Production of the enzyme is constitutive in a wild-type strain growing in media poor in sulphur, but inducible in the same strain growing in sulphur-rich media. Induction of tyrosinase is, however, obligatory in two mutant strains carrying mutations in the regulatory genes already mentioned[108]. These observations, together with a number of demonstrations of the isolation of so-called 'semi-constitutive' mutant strains in which the production of a variety of enzymes is considerable in the absence of inducer, but enhanced by the presence of an inducer, indicates that in some instances regulation of enzyme synthesis may be considerably more complicated than the situations described above. Furthermore, although a number of biosynthetic sequences in bacteria are known to be controlled by 'clusters' of closely linked genes, the genes controlling some enzyme sequences, especially in *Aspergillus* and *Neurospora* are certainly *not* collected into 'clusters' and it would be interesting to determine whether such sequences are under the regulatory influence of a single operator.

Constitutive strains of organisms typically producing particular enzymes inducibly and 'derepressed' strains of *E. coli*, analogous to the mutants no longer repressed by tryptophan, have been reported[12,100,120,123,133]. Detailed genetic study of these strains has not yet been carried out, but it is probable that the observed phenotypic behaviour is due to mutations affecting regulatory mechanisms of the type described above.

ROLE OF NUCLEIC ACIDS IN PROTEIN SYNTHESIS

It must now be accepted as established that proteins consist basically of a linear, or series of connected linear arrangements of amino acids, the

sequence of which, together with the secondary and tertiary configuration adopted, determines the biological specificity of the protein. The mechanism by which the amino acids are arranged in order within the molecule is still largely unknown, but has been the subject of a great deal of speculation. The most widely accepted hypothesis is that amino acids, after prior activation and attachment to s-RNA, are arranged in correct linear order on a template, followed by formation of peptide linkages between adjacent amino acids and release of the completed protein from the template. This hypothesis infers that the templates must be macromolecules capable of determining, in an unambiguous fashion, the amino acid sequences of the great variety of different proteins formed in a single cell. Of the macromolecules found in living organisms the most likely candidates for the role of template are the nucleic acids.

The Role of DNA in Protein Synthesis

Work with transformation systems in bacteria has established that DNA is the carrier of genetic information in these organisms[140]. A virtually pure preparation of DNA from a strain of pneumococcus able to synthesize a specific polysaccharide, when added to a culture of a strain unable to bring about the formation of this substance, conveys to the recipient cell the required information for polysaccharide synthesis. Presumably the information transferred is that required for the synthesis of a specific enzyme implicated in the formation of the polysaccharide. A demonstration of the conveyance, by transformation, of the information for synthesis of specific enzymes mannitol phosphate dehydrogenase, amylomaltase and alkaline phosphatase has since been achieved[134,141,142].

That information for protein synthesis is carried by DNA is also indicated by studies on bacteriophages infecting and lysing *E. coli*, with the liberation of a considerable number of new bacteriophage particles. The infecting bacteriophage particle consists of a DNA 'core' surrounded by a protein sheath and provided with a tail, by which the particle attaches itself to the host bacterium. On infection DNA is injected into the host, but the protein largely remains behind outside the host cell. The DNA carries information for the synthesis of new bacteriophage particles, including the protein components[143]. Infection of *E. coli* by bacteriophage T2 also results in the appearance within the host cell of a number of enzymes not found in uninfected cells. These enzymes are implicated, among other functions, in the synthesis of 5-hydroxymethylcytosine, found in the bacteriophage DNA but not in the host DNA. It is probable that the 'new' enzymes are derived from information carried by the DNA of the infecting bacteriophage particle[144].

These, and other studies[145] focused attention on the primary role of DNA in the determination of protein structure. The simplest idea relating the two is that DNA itself is the template on which protein is formed. Such a relationship would imply that protein is synthesized exclusively in the nucleus. While there is no doubt that protein is formed in the nucleus of higher organisms[146] incontrovertible evidence, already discussed, shows that the bulk of cytoplasmic protein is synthesized in the extranuclear RNP particles. The alternative to a DNA template is a situation in which an

intermediary exists between DNA and protein, the information residing in DNA being transferred to the intermediary which acts as the template.

Many observations, principally on lower organisms, suggest that DNA plays only an indirect role in protein synthesis. Enucleate portions of the marine alga *Acetabularia mediterranea* incorporate amino acids into protein and display increases in total protein and activities of the specific enzymes, aldolase, invertase and phosphorylase. On the other hand, synthesis of acid phosphatase ceases shortly after enucleation[147,148]. Enucleated *Amoeba proteus* incorporates amino acids into protein, although net synthesis cannot be demonstrated since enucleated organisms lose the ability to feed, some protein disappears and other physiological changes occur[147,148]. Furthermore, mammalian reticulocytes, which are devoid of nuclei, synthesize considerable quantities of the protein haemoglobin[4]. Certain experiments with bacterial cells appear to substantiate these results, though most show that continued DNA synthesis is not a requisite of protein synthesis, rather than that DNA is not directly implicated[35,147].

Results at variance with the conclusion that DNA is not directly implicated in protein synthesis have been obtained using *E. coli* labelled with radioactive ^{32}P. In ^{32}P-labelled cells stored at $-196°C$ metabolism is arrested, but ^{32}P decay continues. By differentially labelling cells in their DNA the conclusion was reached that capacity to synthesize the enzymes β-galactosidase and D-serine deaminase are lost at the same rate as loss of viability and the decrease is due to transmutation of ^{32}P atoms in the DNA, leading to breaks in the 'backbone' of DNA molecules. A feature of these results is that they suggest that the integrity of the whole genome is necessary for enzyme synthesis since it is not known whether, or to what extent, the ^{32}P disintegrations occur in the regions of the chromosome controlling the structure of the enzymes actually studied[149]. This conclusion has been criticized[150] on the grounds that synthesis of the enzymes studied is repressed by metabolites accumulating in non-viable cells and that the study of enzymes not subject to such repression or alternatively the omission of an energy source leads to retention of appreciable capacity to synthesize enzymes even after viability has decreased to very low values.

A more clear-cut demonstration of the necessity for the integrity of the gene for protein synthesis was provided by the demonstration that decay of ^{32}P after transfer of ^{32}P-labelled genetic material determining β-galactosidase structure to an unlabelled recipient cell was accompanied by loss of ability to synthesize β-galactosidase[151].

The Role of RNA in Protein Synthesis

Recent work on the mechanism of protein synthesis has implicated the ribosomes, rich in RNA, as the site of synthesis. Furthermore s-RNA acts as a carrier of activated amino acids. Earlier investigations had already implicated RNA in protein synthesis, since the study of the distribution of RNA in different tissues led to recognition of a positive correlation between high RNA content and capacity for rapid synthesis of protein. Within certain limits the correlation is particularly striking in bacterial cells growing at different rates[35]. Other experiments with bacteria and yeast, including

those involving the witholding of RNA precursors, administration of purine and pyrimidine analogues and the action of ribonuclease all suggested that RNA played some part in protein synthesis[35,147]. However, the interpretation of some of these experiments is by no means unequivocal[35]. Moreover the majority were conducted before the role of s-RNA was appreciated and consequently do not provide incontrovertible proof that high molecular weight RNA is involved. In fact, it has recently been shown that ribonuclease, which has a profound effect on amino acid incorporation in onion root tip cells[147] removes s-RNA but not ribosomal RNA[152].

The adoption of the template hypothesis, the demonstration that DNA seemed to be only indirectly concerned in protein synthesis (at least in some organisms) and a knowledge of the role of ribosomes in protein formation led to the widely accepted view that ribosomal RNA is the template on which amino acids are ranged before incorporation into protein. Such a belief implies the DNA-directed synthesis in the nucleus of some (if not all) cytoplasmic RNA, followed by its migration into the cytoplasm and incorporation into the site of protein synthesis. In addition the information for determination of amino acid sequences must be transferred from DNA to RNA. Various suggestions as to how this might be achieved have been made[144] but all depend on the assumption that the information resides in the sequence of bases in DNA and is conveyed in a specific (though as yet unknown) manner to RNA synthesized in juxtaposition to DNA.

Evidence that synthesis of cytoplasmic RNA occurs in the nucleus has been derived from observations involving incorporation of radioactive RNA precursors, followed by detection of the site of incorporation by radioautography. Investigations with enucleate *Amoeba proteus* and enucleate portions of *Acetabularia* yielded somewhat contradictory results[147,148]. The study of *Acanthamoeba*, an amoeba which can be grown in a defined culture medium, so removing some of the ambiguities attending the investigation of other amoebae, showed that enucleate portions failed to incorporate a number of ^{14}C labelled precursors into RNA[148].

The relationship between the nucleus and RNA synthesis has recently been examined in cells of *Neurospora crassa* exposed to radioactive precursors of protein and RNA. After exposure to the desired precursor the organism was subjected to centrifugal forces applied along the length of the cells[153]. This procedure causes a separation of cell organelles into layers, the fractions being (in decreasing order of density): glycogen granules, ergastoplasm (microsomes), mitochondria, nuclei, cytoplasmic sap and fat. Subsequent micro-radioautography allowed identification of the main site of incorporation of RNA and protein precursors. Following a 1 minute exposure to tritiated uridine (a RNA precursor) incorporation occurred exclusively in the nuclei. In 'pulse' experiments short exposure to tritiated uridine followed by unlabelled uridine resulted in label initially incorporated into the nuclei passing mainly into microsomes. The use of tritiated leucine also allowed the demonstration that exposure of a few seconds' duration led to incorporation into protein located mainly in microsomes. These observations show conclusively that the nucleus is the sole site of RNA synthesis in *Neurospora*. Similar conclusions have been reached in studies of higher plant and animal material[148].

Definitive experiments have not yet been conducted with bacteria. Preliminary investigation suggests that RNA synthesis may be associated with the nuclear bodies[154], although the precise interpretation of these experiments is not yet clear. An *in vitro* incorporation of ribonucleotides into RNA and, in some instances, net synthesis of RNA, is catalysed by enzyme extracts from a variety of bacteria. These systems, besides being dependent on ATP, CTP, GTP and UTP for maximum activity, require the presence of a DNA 'primer' for incorporation, which occurs in non-terminal positions in the RNA, showing conclusively that they are quite different to the enzyme responsible for attachment of terminal nucleotides to s-RNA[155,156]. An extract of *Micrococcus lysodeikticus* formed, in the presence of one of a number of different DNA preparations, RNA differing in base sequence and with an over-all base composition corresponding to the DNA 'primer'[156]. Of course, it is not yet possible to determine the precise base sequence of nucleic acids and so compare that of the 'primer' with the newly-formed RNA, but it should prove possible to apply a test for base complementarity (see p. 133) and possibly to devise a test to determine whether the RNA is biologically functional.

Experiments with tobacco mosaic virus (TMV), a plant virus which contains protein and RNA, but no DNA, have shown that RNA can carry at least part of the information required for the synthesis of protein. The RNA and protein of TMV can be separated chemically and the RNA alone is infective. Infection of a suitable host in this way results in the formation of complete virus particles. Several strains of TMV, differing in the structure of the protein component, are known. It is possible, after separating the RNA and protein components of strain A and strain B, to reconstitute them as 'hybrids'—the RNA of strain A with the protein of B (and vice versa). These reconstituted TMV particles are infective, and re-isolation of virus from the host plant has shown that the protein synthesized is that corresponding to the RNA and *not* to the protein of the 'hybrid'. The protein introduced with the 'hybrid' is not replicated[157].

Treatment of the RNA with nitrous acid replaces free $-NH_2$ groups in the nucleotide bases by $=O$, without damage to the 'skeleton' of the RNA molecule. Thus adenine is converted to hypoxanthine, guanine to xanthine and cytosine to uracil[157]. Nitrous acid treatment leads to the formation of stable mutants differing recognizably from TMV as a result of behaviour in suitable host plants. The protein associated with several spontaneous and nitrous acid-induced mutants of TMV differs from that of the original TMV in amino acid composition[158,159]. Thus, in an RNA-virus, information exists for the ordering of an amino acid sequence in protein, and alteration of the nucleotides in the RNA can lead to changes in the structure of the protein component.

Reference has already been made to the fact that introduction of the z^+ allele, the active form of the gene (z) determining the structure of β-galactosidase, into a cell carrying the z^- allele resulted in the formation of β-galactosidase at a constant rate and without detectable lag. Furthermore if the z^+ allele is labelled with ^{32}P before mating with unlabelled recipient cells and the resulting zygotes stored at $-196°C$ to allow ^{32}P decay, so destroying the integrity of the gene DNA, a situation approximating to

removal of the functional gene is obtained. These labelled zygotes suffer a progressive decrease in enzyme forming capacity until, after 10 days storage, ability to synthesize β-galactosidase has virtually disappeared, although the synthetic ability of unlabelled control zygotes is unimpaired[151]. These observations, taken together, suggest that if an intermediary (RNA?) mediates between the gene and the completed protein its formation is very rapid and further it is metabolically unstable and quickly disappears on loss of structural integrity of the gene.

In contrast to these observations ribosomal RNA, widely believed to be the template specifying amino acid sequences, is metabolically stable[35,160]. Furthermore the nucleotide composition of ribosomal RNA does not reflect the nucleotide composition of DNA. An attempt to resolve this paradoxical situation led to the hypothesis that ribosomes are unspecialized structures which receive information from the gene in the form of an unstable 'messenger'[44]. Recently evidence for the formation of such an unstable RNA intermediary has been obtained as a result of study of the changes occurring in bacterial host cells infected with bacteriophage. Within a few minutes of infection a newly formed, metabolically unstable RNA fraction appears in the host cell. This RNA fraction can be distinguished from that of uninfected host cells by its nucleotide composition which corresponds to that of the DNA of the infecting bacteriophage[143]. Furthermore, the RNA formed on infection with bacteriophage T2 can be distinguished from that formed with T7[161]. That the 'new' RNA (for convenience termed 'T2-RNA') is intimately connected with replication of the bacteriophage is shown by the observation that treatment of infected *E. coli* spheroplasts with ribonuclease during the first 10 minutes after infection with T2 prevents the appearance of bacteriophage particles[162]. It is believed that the formation of T2-RNA is associated with the synthesis of bacteriophage proteins and possibly with the formation of specific enzymes required for production of bacteriophage components.

The T2-RNA is associated with the ribosomes of the host, although synthesis of new ribosomes stops on infection. This can only mean that the T2-RNA becomes associated with existing ribosomes, from which it can be detached by lowering the magnesium ion concentration or during centrifugation in a caesium chloride gradient. Neither of these procedures removes ribosomal RNA from ribosomes[44,163]. It was suggested that T2-RNA represents 'messenger' RNA formed under the influence of newly introduced (bacteriophage) DNA. A comparable RNA fraction, having a high turnover rate (*i.e.* metabolically unstable) and the property of reversible attachment to ribosomes has been demonstrated in normal (uninfected) cells[164] and a similar RNA fraction has been detected in yeast cells[165]. As already stated a proportion of the 70 S ribosomes of *E. coli* do not dissociate on lowering the magnesium ion concentration; these ribosomes are particularly active in *in vitro* incorporation of amino acids into protein[43], and much of the 'messenger' RNA appears to be associated with undissociable 70 S ribosomes[44,164], although an association with smaller particles has been claimed[163]. Besides differences in composition ribosomal and 'messenger' RNAs differ in particle size. After isolation by phenol extraction the former yields 23 S and 16 S particles, whereas the latter consists of 8 S particles[163,164].

Native DNA exists (with certain exceptions) as a double-stranded helix, the components of which can be separated by heating, and reconstituted by cooling under carefully controlled conditions[166]. The ability of two strands to recombine by hydrogen-bonded base pairing (see below) also extends to synthetic polynucleotides[166], and has been used as a test to detect base complementarity between DNA of bacteriophage T2 and the T2-RNA formed in *E. coli* cells. Under appropriate conditions of cooling, T2-DNA that had been heated to separate the two strands of the double helix and then mixed with T2-RNA from infected *E. coli* cells, forms 'hybrid' molecules between a DNA strand and the T2-RNA[167]. The formation of the 'hybrid' is specific for T2-DNA and T2-RNA and it was concluded that the base sequence of T2-RNA is complementary to bacteriophage T2-DNA, but not complementary to DNA from bacteria or from bacteriophage T5.

These observations constitute the first demonstration that introduction of specific DNA into a cell results in the formation of specific RNA of complementary base sequence, the existence of which had already been postulated as an intermediary carrier of information between the gene (DNA) and the site of protein synthesis. Furthermore this RNA is found attached to ribosomes—the expected location of a template RNA. It must be borne in mind, however, that some of the RNA formed on infection with bacteriophage appears to be metabolically stable and the proportion of stable RNA increases with time after infection. Further, inhibition of RNA synthesis, modification of RNA by administration of purine or pyrimidine analogues or treatment with ribonuclease 5 to 10 minutes (or longer) after infection does not prevent continued synthesis of bacteriophage proteins[162,168,169]. It would appear from these results that 'messenger' RNA may, in some way, become stabilized and no longer subject to the rapid turnover observed during the first few minutes of infection.

The Coding Problem

Deoxyribonucleic acid consists of linear chains of deoxyribonucleotides, the deoxyribose sugar of adjacent nucleotides being linked by phosphate bridges. To each sugar moiety is attached a purine or pyrimidine base, the most important quantitatively being the two purines, adenine and guanine and the two pyrimidines, thymine and cytosine. Other bases occur in much smaller amounts. It is accepted that, with certain rare exceptions, DNA exists in nature as a double helix, and two chains bound together by hydrogen bonds between opposite bases, adenine always being paired with thymine and guanine with cytosine—the so-called 'Watson-Crick' model[166].

If DNA specifies the linear arrangement of amino acids in protein molecules then the information must reside in the order of the four bases along the linear deoxyribosephosphate backbone of the DNA, a certain combination of bases specifying a particular amino acid. That is, the information governing amino acid sequence is *coded* in the DNA base sequence. The coding problem can be reduced to the problem of specifying the twenty amino acids commonly found in proteins in terms of four main bases, an exercise which has engaged the attention of many workers, but need not be discussed fully here[19,144,145,170,171]. As an example may be

mentioned the code of Crick, Griffith and Orgel[170] based on a series of non-overlapping base triplets, each amino acid being represented by three bases. Any triplet that could be made by reading the last part of one triplet and the first part of the adjacent triplet could not mean anything in the code. That is to say, if A, B, C and D represent the four bases, and if BAC and DBC represent the triplets denoting two different amino acids, then ACD and CDB could not denote amino acids ('nonsense' triplets). The advantages of such a non-overlapping triplet code are twofold. Firstly, it places no restrictions on adjacent amino acids; secondly, it is independent of having to be read from one end since there is no ambiguity about what constitutes a 'sense' (amino acid determining) triplet. Such codes are termed 'commaless' because they do not require bases to act as 'punctuation' between individual triplets.

If mutation involves substitution of a base in the DNA chain by another base, then a change which leads to a 'nonsense' triplet may then prevent any intact protein being synthesized since a gap would appear in the amino acid sequence. However, a mutation of one 'sense' triplet to a different 'sense' triplet will lead to the replacement of, for instance, lysine by valine, and so may yield either a biologically active protein with altered physico-chemical characteristics or loss of enzymic activity. It will be seen later that mutation can lead to the formation of proteins aberrant as the result of changes in amino acid sequence. A number of proteins having altered physico-chemical and enzymic characteristics as a result of mutation have been studied, but the nature of the amino acid substitutions has not yet been determined.

It has been widely accepted, and appears logical to suppose that the code is likely to be universal among living organisms. However, the DNA of different micro-organisms has widely differing base ratios, *i.e.* the ratio (guanine + cytosine)/(adenine + thymine) ranges from values[172] of about 0·5 to about 2·5, whereas the equivalent ratios for RNA (the thymine of DNA being equated with uracil of RNA) of the same organisms varies very little. Furthermore the relative abundance of the different amino acids in proteins does not vary greatly in different organisms. A number of suggestions have been put forward to explain this discrepancy between DNA base ratios[173], among them the idea that only a part of the DNA base sequence is carrying information for the determination of amino acid sequences, the code is not universal throughout nature, or alternatively, the code is based on fewer than four characters. To circumvent this difficulty codes, usually based on two characters have been devised, allowing wide changes in the base composition without affecting the message conveyed by the code[144,145,174].

If some base sequences in DNA are not implicated in the determination of amino acid sequences in protein, then sequences may serve as 'punctuation' between the individual sequences denoting single amino acids. Although it is frequently assumed that the sole function of DNA sequences is to store information for amino acid sequences, this is by no means certain. For instance, evidence already discussed suggests that, in micro-organisms at least, the *regulation* of enzyme synthesis is under genetic control, and the DNA segments concerned in this function may not be coding protein. Recent evidence also indicates that 'structural' ribosomal RNA may not

comprise the templates on which proteins are assembled. It is likely that base sequences in this RNA are determined by DNA. It has also been shown that the bulk, if not all, of RNA in cells is synthesized in the nucleus. It is likely that base sequences of ribosomal RNA and possibly of s-RNA are determined by DNA, so suggesting the presence of further DNA which is not coding protein.

For spatial and other reasons Crick[170] thought it unlikely that amino acids would be orientated directly on the template and postulated a series of 'adaptor' molecules to which amino acids could become attached, another portion of the 'adaptors' becoming attached, probably by hydrogen bonds, to the triplets of bases on the template. This suggestion was originally made before the significance of s-RNA was fully realized. The discovery that s-RNA is involved in transfer of amino acids to ribosomes provides, in recognizable form, the postulated 'adaptor' molecules.

The overall picture emerging from a consideration of available experimental evidence and the theoretical treatment of coding is one involving storage (and at nuclear division, the replication) of information specifying amino acid sequences in the base sequence of DNA, and the transfer of this information into specific base sequences in 'messenger' RNA, probably involving hydrogen bonding between DNA bases and RNA bases at the time of RNA synthesis. Amino acids could then be orientated on 'messenger' RNA templates, again involving base pairing by hydrogen bond formation between template and s-RNA. After formation of peptide linkages between adjacent amino acids, followed by liberation of the completed protein, the s-RNA would be released. Most of the evidence at present available is in accord with this hypothesis, but recently it has been suggested that s-RNA is not bound to ribosomes by hydrogen bonds, but by covalent linkages[36].

Incorporation of Purine and Pyrimidine Analogues: Experimental Modification of Templates

A wide range of purine and pyrimidine analogues, produced by chemical substitutions in the naturally-occurring bases, can be incorporated into the nucleic acids of micro-organisms, plant and bacterial viruses, and both normal and tumour tissues of animals. Incorporation is accompanied by changes in biological properties of the cells or viruses concerned[86,175]. Incorporation of a purine or pyrimidine analogue leads to a corresponding decrease in the amount of a natural purine or pyrimidine found in nucleic acid. Thus it has become usual to speak of 'replacement' of a nucleic acid base by an analogue, although this may be an oversimplification, since it was recently shown that replacement of thymine by bromouracil in the DNA of *E. coli* was accompanied by a marked change in the distribution of other DNA bases[176]. Experiments with plant and bacterial viruses have yielded convincing evidence that incorporation of purine and pyrimidine analogues can lead to the synthesis of biologically impaired nucleic acids[86]. Space does not permit an exhaustive discussion of the effects of substituted purines and pyrimidines on micro-organisms; discussion will be confined to an examination of available evidence relating to one aspect, namely whether, if RNA is the template controlling the sequence of amino acids in proteins,

experimental modification of RNA by growth in the presence of analogues can lead to demonstrable changes in the proteins synthesized, a proposal first put forward by Chantrenne[177].

Azaguanine, an analogue of the purine guanine, is incorporated almost exclusively into the RNA of bacteria[86]. Addition of 8-azaguanine to *Staphylococcus aureus* results in inhibition of the development of induced β-galactosidase activity, the inhibition being complete if the analogue is added with the inducer, but less pronounced if it is added later in the experiment. The inhibitory effect of 8-azaguanine is reversed by guanine, the reversal being complete if guanine is added soon after the analogue, but becomes more difficult and incomplete if addition of guanine is delayed[77].

These observations have been confirmed and extended in an intensive study of the effects of azaguanine on protein synthesis by *Bacillus cereus*. Low concentrations of the analogue completely suppressed synthesis of active penicillinase, while only partially suppressing total protein synthesis, measured by incorporation of radioactive phenylalanine into protein. After 30 minutes' exposure to the analogue incorporation of ^{14}C–labelled phenylalanine recommenced almost immediately on addition of guanosine (the nucleoside derivative of guanine) but there was a considerable delay in the synthesis of active penicillinase. Prolonged exposure to the analogue before addition of guanosine leads to a considerable delay before protein synthesis recommences and complete failure to produce active penicillinase. These observations are consistent with the suggestion that exposure to azaguanine results in synthesis of proteins having impaired biological activity. Labelled azaguanine is incorporated into RNA, but on addition of guanosine radioactive material is excreted into the medium, although the excretion is incomplete[178].

It was suggested that the effect of azaguanine is twofold. Firstly, it is rapidly incorporated into guanine derivatives with a high rate of turnover, so accounting for the immediate yet readily reversible inhibitory effect on protein synthesis. The subsequent discovery of 'messenger' RNA[44], metabolically unstable and therefore constantly renewed, lends support to these views. Secondly, the rapid incorporation into unstable substances is accompanied by a slower, irreversible incorporation into stable guanine derivatives, such as ribosomal RNA. Although the function of ribosomal RNA is unknown these observations suggest it has a definite role in protein synthesis, possibly by acting, by formation of hydrogen bonds, as a supporting structure for 'messenger' RNA. Little is known about the metabolic stability, or otherwise, of s-RNA but azaguanine (and presumably other purine and pyrimidine analogues) can be incorporated into s-RNA[179] though the significance of this cannot yet be assessed.

Direct evidence that incorporation of a purine or pyrimidine analogue into RNA can lead to the formation of proteins possessing altered physicochemical or enzymic properties has recently been obtained. Growth of *E. coli* in the presence of thiouracil leads to incorporation of the analogue into RNA. Under these conditions the β-galactosidase activity of the cells is considerably decreased and the enzyme itself is demonstrably immunologically different to normal β-galactosidase[180]. Under appropriate experimental conditions another uracil analogue, 5-fluorouracil is incorporated

into *E. coli* RNA. During growth in the presence of this analogue no active β-galactosidase or β-glucuronidase is produced. However a protein antigenically related to β-galactosidase, but lacking enzymic activity has been detected in fluorouracil-grown cells. Moreover the alkaline phosphatase formed after growth in the presence of fluorouracil, although posessing normal enzymic activity, is less thermostable than the normal enzyme[181].

These results demonstrate clearly that the presence of 'unnatural' components in the RNA of bacteria result in the formation of proteins either lacking enzymic activity, or possessing physico-chemical characteristics differing from those of the normal enzyme. It is likely that replacement of naturally-occurring bases in a nucleic acid template by analogues provokes errors in amino acid sequences in proteins, leading to observable changes in enzymes. Exposure of growing *E. coli* cells to ^{14}C-labelled fluorouracil results in rapid incorporation of radioactivity into the 'messenger' RNA fraction[181]. This observation supports the view[44,164] that 'messenger' RNA is probably the intermediate carrier of information specifying amino acid sequences and suggests that alteration of this RNA fraction by an analogue leads to modification of proteins by promoting changes in the primary (and indirectly the secondary and tertiary) structure.

Alteration of Protein Structure as a Result of Mutation

The study of artificially-induced mutants of micro-organisms requiring single specific substances for growth led to the formulation of the 'one gene–one enzyme (protein)' hypothesis[145,182]. In its simplest form this hypothesis states that the synthesis of a single enzyme (or specific protein) is controlled by a single gene, mutation within the gene leading to a change in the protein which may amount to complete loss of biological activity. As already pointed out mutation might be expected to lead to substitution of one amino acid by another in the primary structure of a protein. The first definite demonstration that such substitutions can occur as a result of a gene mutation stems from the study of sickle-cell haemoglobin in humans, the presence of which is known to be due to mutation at a single locus. The investigation of peptides derived by partial hydrolysis of normal (A) and sickle-cell (S) haemoglobins revealed that a glutamic acid residue present in haemoglobin A is replaced by a valine residue in haemoglobin S. Comparable amino acid substitutions (although involving different amino acids and different positions in the molecule) have been described in other abnormal haemoglobins[145,171]. The whole haemoglobin molecule is formed from four sub-units, two identical α-chains and two identical β-chains. Both α- and β-chains are polypeptides, the structure of each being determined by a separate gene. The genes are not closely linked[145]. It is known that a number of enzymes consist of more than one type of polypeptide; future investigation will probably show that, in all such instances, the structure of each polypeptide chain is controlled by a separate gene.

Comparable studies are at present being made on bacterial and bacteriophage proteins. These systems lend themselves to very detailed genetic mapping and, although the detection of amino acid substitutions is only just beginning, it is expected that ultimately investigations of this type will lead

to mapping of defects in proteins and a correlation of such defects with changes in the genetic material[171,183].

In micro-organisms several cases of mutants producing enzymes with changed physico-chemical characteristics have been studied. Changes so far detected include increased thermolability, altered affinity for substrates, sensitivity to heavy metal ions and altered electrophoretic mobility[125,171,182,184]. Mutants which appear to have lost a particular enzymic activity completely sometimes produce immunologically cross-reacting material. Immunological cross-reaction of this type denotes pronounced similarity between the enzyme (antigen) and the cross-reacting material, suggesting that the latter is an altered protein, closely resembling the enzyme itself, and produced as a result of mutation. These instances of evidence of the production of altered proteins have been described mainly in the fungus *Neurospora crassa*, in *E. coli* and bacteriophages. Unfortunately space does not permit a full discussion of the many interesting biochemical and genetical investigations conducted on such systems and the reader is referred to the excellent reviews cited above.

Modifications in the tryptophan synthetase systems of *E. coli* and *N. crassa* have been intensively studied in mutants requiring tryptophan for growth. The wild-type *E. coli* enzyme is a compound protein consisting of two components, *A* and *B*, which can be separated by appropriate treatment and reconstituted by mixing the two fractions. The complex catalyses three separate reactions:

$$\text{Indole} + \text{serine} \rightarrow \text{tryptophan} \tag{3.3}$$

$$\text{Indoleglycerol phosphate} \rightarrow \text{indole} + \text{triose phosphate} \tag{3.4}$$

$$\text{Indoleglycerol phosphate} + \text{serine} \rightarrow \text{tryptophan} + \text{triose phosphate} \tag{3.5}$$

Neither *A* or *B* components alone are active in promoting these reactions at appreciable rates. Each can be assayed separately by addition of an excess of the other component and specific antisera can be prepared against each component. The synthesis of *A* and *B* components is controlled by two separate genes which are very closely linked.

A series of mutants, all lacking enzymically active tryptophan synthetase complex were examined for ability of their *A* and *B* components to catalyse reactions (3.3), (3.4) and (3.5) in the presence of the opposite component from wild type organism. A number of mutants, defective in component *A*, formed a protein which still reacted with anti-*A* serum and (in conjunction with normal *B* component) promoted reaction (3.3), but not reactions (3.4) and (3.5). Other *A* mutants appeared to lack component *A* completely, since there was no detectable immunological reaction with antiserum, nor detectable enzymic activity in the presence of normal *B* component. Similarly, another series of mutants produced altered component *B*. A number of these mutants lacked this component completely since they displayed neither enzymic nor serological reactions. The remainder synthesized a modified *B* component which catalysed only reaction (3.4) and reacted with anti-*B* serum. The tryptophan synthetase of *N. crassa* cannot be separated into two components, as in *E. coli*. Several mutants lacked detectable tryptophan synthetase activity, but formed cross-reacting material (CRM) capable of reacting with antiserum produced against normal enzyme[125,171,184].

Certain *E. coli* mutants lacking the ability to form active β-galactosidase (z) produced material which cross-reacted with anti-β-galactosidase serum. Diploids containing z^+ and z^- alleles in a common cytoplasm produced functional β-galactosidase and CRM simultaneously and the production of both was affected in an identical fashion in the presence of different concentrations of inducers[136].

Of considerable interest in a consideration of genetic determination of enzyme structure and the transfer of information from the gene to the actual site of protein synthesis is the observation that, when two types of nuclei, each carrying a mutation of the gene controlling the structure of a particular enzyme, are combined in a common cytoplasm they sometimes interact with the result that detectable enzyme activity is produced, even though neither mutant strain itself forms active enzyme. This behaviour has been described in heterocaryons involving two glutamic dehydrogenase-deficient strains of *N. crassa* and certain pairs of adenylosuccinase-deficient mutants of the same organism[145,171,184]. Similar observations have been made with other organisms. These are unexpected results since each mutant participating does not form active enzyme, and in the heterocaryons it might be expected that two altered proteins could be formed, but that the heterocaryon would remain devoid of activity. In fact, when enzyme complementation of this type occurs the enzyme levels observed are never more than 25 per cent of wild type levels, and often considerably less than this. Furthermore, the glutamic dehydrogenases of two *Neurospora* heterocaryons were demonstrably abnormal[184], as was the adenylosuccinase formed by some heterocaryons between pairs of mutants lacking adenylosuccinase activity. However the enzyme produced by two other adenylosuccinase-deficient mutants did not differ detectably from normal (wild-type) enzyme by the tests employed[185]. A similar type of interaction has been demonstrated *in vitro*, using two adenylosuccinase-deficient mutants of *N. crassa* which displayed complementation in a heterocaryon. Extracts of cells of these strains, neither of which possessed enzyme activity, displayed adenylosuccinase activity when mixed together[184]. Tryptophan-dependent mutants of *N. crassa*, all allelic, have been examined for their ability to complement in heterocaryons between pairs of mutant strains. Complementation occurred only when both participants formed CRM and the resulting heterocaryons contained three components related to the tryptophan synthetase system: the CRM characteristic of each participant and a protein possessing tryptophan synthetase activity[125].

The mechanism of intragenic complementation of this type is obscure, but several possible explanations have been proposed, including 'recombination' between portions of defective RNA templates, 'recombination' between two defective polypeptide chains or, where an enzyme consists of two or more identical sub-units, combination of polypeptides defective in different ways to give a functional, though abnormal, enzyme[145,171,182,184]. Greater understanding of this phenomenon must await further experimentation. A full understanding of gene–enzyme relationships also awaits elucidation of the mechanism of action of suppressor mutations. These lead to partial or complete recovery of an enzymic activity lost as a result of a previous mutation at a different locus. Space does not permit a

discussion of suppressors, but their effects are described in several recent reviews[125,171,184].

CONCLUSIONS

In the Introduction to this account six questions were posed. Although the study of the biochemical mechanism and genetic control of protein biosynthesis has been among the most active and exciting areas of biological research during the past decade the fact that few of these questions can be adequately answered is a measure of our ignorance of the mode of synthesis of these all-important components of all living organisms. Many of the questions posed remain the subject of intensive speculation.

Significant progress in breaking the code relating base sequences in nucleic acid to amino acid sequences in proteins will probably come through continuation of current research on 'simple' entities such as plant viruses and bacteriophages containing only one or a few proteins. Another aspect of the problem at present under active investigation[31] is that of base sequences in s-RNA molecules. As Hoagland[4] has pointed out these molecules appear to consist of sequences of undue length for the function they perform, yet the effect of nitrous acid on s-RNA suggests that virtually the whole sequence is necessary for functional integrity[21].

The apparently indirect role of DNA in protein synthesis in *Acetabularia* and the necessity for the continued presence of the functional gene to assure the synthesis of protein in bacteria is a paradox which remains unresolved. Reticulocytes, which form protein in the absence of a nucleus may represent a special case since they synthesize mainly a single protein, namely haemoglobin. It is possible that in bacteria recourse to the 'master copy' of the information (the gene) must be made more frequently than in higher organisms, owing to differences in stability of the templates. In this context it may be noted that cessation of enzyme synthesis on addition of an external repressor metabolite is very rapid, a fact in accord with the observed metabolic instability of 'messenger' RNA, since if templates were stable enzyme synthesis might be expected to continue after addition of the repressor metabolite as a result of continuing function of pre-existing templates. The apparent metabolic instability of 'messenger' RNA, here assumed to be synomynous with the template, may also account, at least in part, for the failure to observe net synthesis of protein in many subcellular preparations.

The so-called 'sequence' hypothesis, which states that the amino acid sequence of a protein is coded in the nucleotide sequence of DNA[170], implies that the genetic material carries information specifying only the primary structure of a protein and that the secondary and tertiary configuration adopted is a direct consequence of the primary amino acid sequence. Evidence to support or refute this corollary of the sequence hypothesis is scanty and stems chiefly from physico-chemical investigations entailing the use of agents which destroy secondary and tertiary structure (and usually enzymic activity), and a study of the reacquisition of specific biological properties on removal of the agent and restoration of physiological conditions. The data from such investigations cannot be discussed fully, but indicate that, in some instances, biological activity, and therefore presumably some resemblance of the native state of the protein, can be regained[3].

However, it is at present impossible to decide whether folding and superfolding of polypeptide chains is directed or spontaneous.

So far little has been said of the way in which the template operates. The experimental investigation of this aspect of protein synthesis has commenced. Two extremes may be considered: (*a*) that all the amino acid components of a polypeptide chain may be arranged in order on the template, followed by more or less simultaneous forging of peptide linkages and release of the completed chain from the template, and (*b*) that a polypeptide chain 'grows' from one end by the sequential addition of amino acids until the chain is complete. Clearly possibility (*b*) may be extended to yield a third situation where 'growth' of the chain commences at two or more points on the template. Evidence supporting mechanism (*b*) has been obtained during study of haemoglobin synthesis in rabbit reticulocytes. The *N*-terminal amino acid of haemoglobin is valine, and an investigation of the distribution of radioactivity in the *N*-terminal valine and in the valine residues of the rest of the haemoglobin chain after exposure of ribosomes previously labelled with ^{14}C-labelled valine to unlabelled valine and vice versa allowed the conclusion that the haemoglobin chain is synthesized stepwise, commencing at the *N*-terminal end, and that ribosomes contain a population of incomplete haemoglobin chains of differing lengths[186]. The elegant experimental approach of Dintzis[187] led to the same conclusion.

Much remains to be learned about the new concept of 'regulator' and 'operator' genes. Substantial evidence supporting the concept exists only for the control of the β-galactosidase system of *E. coli*, although some supporting evidence comes from studies of other systems. Since the bulk of this article was written Jacob and Monod[188] have presented a detailed examination of the genetic regulation of enzyme synthesis. In addition, control of alkaline phosphatase in *E. coli* has been shown to be under the control of two 'regulator' genes, one closely linked to the 'structural' gene, the other distant from the 'structural' gene[189]. Formation of this enzyme in the wild type strain is repressed by inorganic phosphate[132] and mutations in either of the 'regulator' genes can lead to constitutive production, the repressive effect of orthophosphate being abolished[189]. At present there is no indication of how the 'operator' functions, nor of the precise nature of the repressor or the nature of the interaction between the repressor product of regulatory genes and inducers[188].

Berg and his colleagues[190–193] have also recently published the results of an intensive study of the formation of s-RNA–amino acid complexes in *E. coli*. Since amino acid activating enzymes are also responsible for the transfer of amino acyl adenylates (activated amino acids) to s-RNA they suggest that these enzymes should be termed amino acyl RNA synthetases.

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4

PREDACIOUS FUNGI AND THE CONTROL OF EELWORMS

C. L. DUDDINGTON

INTRODUCTION

THE predacious fungi are a somewhat heterogeneous group of microfungi that prey on microscopic animals: Protozoa, rotifers and, particularly, nematode worms (eelworms). Most of the nematode-attacking species fall within the Order Moniliales (Hyphomycetes) of the class Fungi Imperfecti. This Order contains fungi with septate, colourless hyphae, asexual reproduction by means of spores (conidia) that are not contained in a sporangium, and no known sexual stage. A number of species, however, belong to the Zoopagales, an order of Phycomcetes in which the hyphae are non-septate and sexual reproduction by the union of a pair of equal or unequal gametangia may or may not occur. A few nematode-attacking fungi are referred to other groups.

We can recognize two distinct types of predacious activity among the predacious fungi that attack nematodes: the eelworm-trappers and the endozoic parasites. The fungi of the first group capture nematodes alive by means of various trapping devices: adhesive networks attached to the mycelium, adhesive knobs set on short lateral branches, hyphal rings that constrict about an eelworm's body and hold it, and so on. The endozoic parasites, on the other hand, usually attack their prey through the medium of spores that stick to an eelworm's body on contact, the spores germinating and forming germ tubes that penetrate the body of the eelworm and give rise to an extensive mycelium within it. At least one of the endozoic parasites, however, has spores that are swallowed by the host.

It is a matter of argument whether the term 'predacious' can strictly be applied to the endozoic parasites of eelworms. It is, however, convenient to include them in the ecological group of predacious fungi, and the species with sticky spores, by analogy with the predacious species with sticky eelworm traps, may be regarded as being predacious during their reproductive phase. It is difficult to draw a hard and fast line between a species with sticky hyphae and one with sticky spores, and at least one species, *Nematoctonus haptocladus* Drechsler[1], has sticky traps on its mycelium as well as sticky spores. I shall therefore follow the usual custom of using the term 'predacious' for both types of fungi.

The eelworms that are victimized by the predacious fungi are mainly the free-living nematodes that abound in soil, in rotting organic matter of most kinds, in dung, in moss cushions and, in fact, almost everywhere. They are all small, measuring from about 100 μ to rather more than 1 mm in length, but they are strong for their size and very active. They move by a vigorous side-to-side threshing action of their bodies, and not by the peristaltic type

of movement found in the earthworms. There is experimental evidence, however, that the nematode-trapping species of predacious fungi can also capture and kill some of the plant parasitic nematodes that are serious pests of crops, as well as some of the smaller nematodes parasitic in animals[2–7]. What part, if any, the predacious fungi play in Nature in reducing the numbers of pathogenic nematodes in the soil is still a matter of conjecture: this aspect of their activity will be considered later.

The history of our knowledge of predacious fungi has been summarized by Drechsler[8]. Predacious fungi attacking nematode worms have been known since 1874, when Lohde described a fungus parasitic in an eelworm; although his description of the fungus was somewhat scanty, it is usually assumed that the species concerned was *Harposporium anguillulae*. This fungus has been recorded many times since: it is, in fact, one of the commonest of all the fungi that are internally parasitic in eelworms.

The first recorded observation of a predacious fungus trapping eelworms came in 1888, when Zopf[9], in a classical paper, described the action of the adhesive networks of *Arthrobotrys oligospora*. This fungus was first described by Fresenius 36 years earlier, but its taste for eelworms had escaped notice until Zopf caught it *flagrante delicto*. It is true that Woronin in 1870 had noticed that the spores of *A. oligospora*, on germination, sometimes gave rise to hyphal networks, but he did not suspect the reason for the formation of these curious structures.

There are other, even more remarkable, instances of a fungus being known for many years without anybody being aware that it was an eelworm-trapper. *Arthrobotrys superba* was first described by Corda in 1839, but it was not until 1937 that Drechsler[10] showed that it captured eelworms in sticky networks in the same way as *A. oligospora*. In the same paper Drechsler pointed out that *Dactyella ellipsospora* captured eelworms by means of sticky knobs at the ends of short lateral branches of the mycelium, and that *Dactylaria candida* also trapped eelworms both by sticky knobs and by non-constricting hyphal rings. Both these fungi had been known for many years without their predacious activities being suspected.

It may seem strange that a fungus could be known for more than half a century without its predacious habit being detected, but there is a reason for it. Predacious Hyphomycetes grown in the absence of eelworms do not usually form the characteristic eelworm traps by which their nature becomes obvious: in pure culture they grow like ordinary moulds. It is not normal mycological practice to allow fungus cultures to become a playground for eelworms or, for that matter, any other fauna. To observe predacious fungi in action, cultures must be allowed to run wild, for it is only in the microcosmic jungle that develops in a heavily contaminated culture that predacious fungi show themselves in their true colours. The fact that the predacious nature of *Arthrobotrys oligospora* and others went so long unsuspected must be regarded as a tribute to the technique of the earlier workers rather than as a slur on their powers of observation.

Before 1935, then, the predacious fungi as an ecological group were virtually unknown, although *Arthrobotrys oligospora* and its eelworm traps had been studied by a number of workers, and one or two other predacious fungi had been discovered. Few people were interested in them, or had even

heard of them; they were looked on as mycological curiosities, nobody dreaming that they were in fact extremely common fungi. They remained unseen because nobody looked for them.

In the early 1930's Drechsler, in the U. S. A., turned his attention to the predacious fungi. His work on species of *Pythium* occurring in the soil led him to plate out pieces of decaying plant roots. These were ideal material for supplying predacious fungi, for they contained nematodes galore, as well as terricolous amoebae and other small animals. When these mixed cultures were supplemented by the addition of pinches of forest leaf-mould they yielded a rich flora of predacious fungi of various kinds. In this way began what has become one of the great researches of mycology: Drechsler, during the past 35 years or so, has not only shown that predacious fungi are a normal part of the fungus flora of soil and decaying organic matter, but has described in great detail a long list of new species. Many of these belong to the Zoopagaceae, a family founded by Drechsler to accommodate Phycomcetes preying on soil amoebae and eelworms, and consisting almost entirely of predacious species. The work of Drechsler has not merely put the predacious fungi on the mycological map: it has supplied the greater part of our total knowledge of these interesting organisms.

THE NEMATODE-TRAPPING HYPHOMYCETES AND PHYCOMYCETES

The nematode-trapping Hyphomycetes are remarkable for the complexity and efficiency of their eelworm traps. These show a degree of morphological specialization unparalleled among the Fungi Imperfecti, most of which are of simple structure.

We can divide the eelworm traps broadly into two types: adhesive traps, that catch eelworms as flies are caught on a fly-paper, and traps that work by mechanical entanglement, without the use of any sticky secretion. Adhesive traps take various forms, of which the most important are:

(*a*) *Sticky networks:* Short lateral branches from the mycelium curl round and join up either with the parent hypha or with other branches, forming complex networks. These are sticky: eelworms that happen to touch them are captured by adhesion, and the death of the captive is followed by the growth of hyphae, from the network, into its body (*Figure 4.1*).

(*b*) *Sticky branches:* The mycelium produces short lateral branches that are sticky. Some of the branches may anastomose to form loops, but complicated systems of networks are not produced (*Figure 4.2*).

(*c*) *Sticky knobs:* Small, spherical or ovoid knobs are formed at the ends of short branches from the mycelium. The knobs, but not the branches that bear them, are sticky and capture eelworms by adhesion (*Figure 4.3*).

There are two principal types of mechanical traps. These are:

(*a*) *Non-constricting rings:* Lateral branches from the mycelium curl round, forming stalked rings (*Figure 4.4*). Eelworms that accidentally push their heads into the rings become wedged in the efforts to force their way through, and so are captured.

(*b*) *Constricting rings:* As in the non-constricting ring, a lateral branch from the mycelium curls round, forming a three-celled ring on a two-celled

stalk (*Figure 4.5*). The three cells that compose the ring are sensitive to touch: if an eelworm pushes its body into a ring, the three cells suddenly swell inwards, closing the ring and so gripping the victim firmly.

Besides the eelworm traps of the nematode-trapping Hyphomycetes, mention must be made of the sticky mechanism found in the Zoopagales. These fungi are widely separated taxonomically from the Hyphomycetes, being placed in the Phycomycetes between the Mucorales and the Entomophthorales: they are difficult to relate to other groups, but on the whole their affinities would seem to be with the Mucorales rather than with the highly specialized Entomophthorales. In the Zoopagales that capture eelworms, the whole mycelium appears to be sticky, there being no specialized eelworm traps. An eelworm touching any part of the mycelium is held by a sticky secretion (*Figure 4.6*).

Most of the mycelial Zoopagales capture small amoebae in soil and rotting vegetable matter, but some of the larger species prey on nematodes. The group also includes many species that are internally parasitic in amoebae and other Protozoa.

Among the eelworm-trapping Hyphomycetes the adhesive network is the commonest type of trapping mechanism. Its operation is well illustrated by *Arthrobotrys oligospora*, one of the most widespread of all the predacious fungi. This species appears to be worldwide in its distribution, and is found in habitats of widely differing type: soil, decaying vegetation, dung, moss cushions and, in fact, almost anywhere where free-living nematodes occur in reasonable numbers. It is sometimes said that *A. oligospora* is primarily a dung-inhabiting fungus. This statement is not justified. It is true that it occurs with remarkable frequency in horse dung, but in my experience it is equally common in other habitats. Our present knowledge of the ecology of the predacious fungi is not sufficient to allow generalizations to be made about their habitat preferences, if any: this is a field of research in which more work is badly needed, and which offers great scope for the research worker.

The mycelium of *Arthrobotrys oligospora* consists of sparingly branched hyphae which are usually about 5 μ in diameter. They are regularly septate and, at least in laboratory cultures, they tend to run straight, with their branches diverging at fairly wide angles—a characteristic that applies to most of the predacious Hyphomycetes.

If *Arthrobotrys oligospora* is growing in the presence of nematodes, the hyphae will bear at intervals the tangles of loops by which eelworms are captured. These are formed by short lateral branches that curl round and anastomose with the hypha from which they arose, or with other loops adjacent to them. The result is the formation of complex systems of networks in three dimensions (*Figure 4.1*). When the fungus is growing on a surface, the loops usually stand up vertically, like croquet hoops, and, as they are often oriented at right angles to one another, the effect when seen under the high power of the microscope is rather suggestive of the semicircular canals of the mammalian ear. The hyphae forming the loops are somewhat greater in diameter than those of the rest of the mycelium, and are highly vaculoated.

If a wandering eelworm happens to brush against one of the loops it is held by means of a sticky secretion, produced apparently by the cells of the

loop. This secretion can easily be seen under the microscope as a colourless, treacle-like fluid. Its chemical nature has not been investigated, but as bird-lime—or perhaps one should say eelworm-lime—it is highly effective. Large nematodes, measuring more than a millimeter in length, are securely held, and though they struggle violently, dragging the mycelium of the fungus this way and that as they desperately try to get away, they seldom

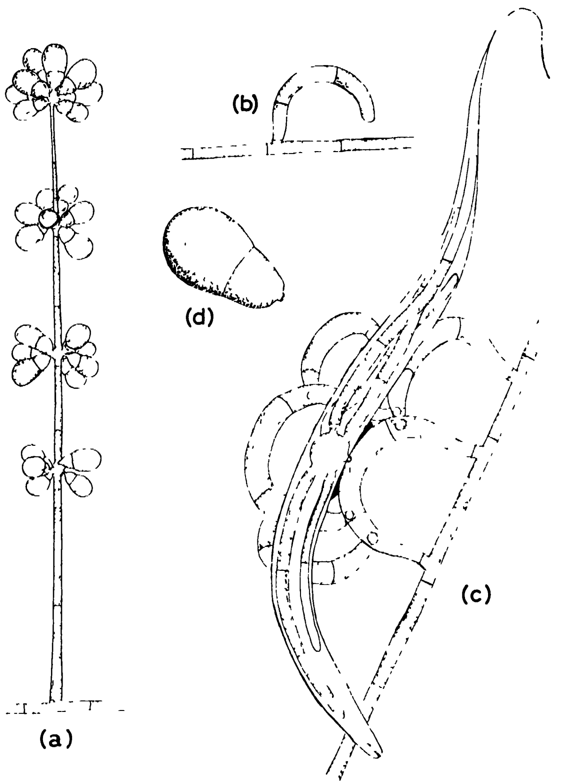

Figure 4.1. Arthrobotrys oligospora
(*a*) erect fertile hypha bearing four whorls of spores, (*b*) lateral branch curling round in the first stage of network formation; (*c*) eelworm captured by a sticky network, showing infection bulb and trophic hyphae in the body of the prey; (*d*) two-celled spore

manage to free themselves. After an eelworm has been captured it struggles violently for a time. Gradually, however, its contortions become less energetic, and periods of quiescence alternate with struggles that become increasingly feeble. Finally the captive remains motionless and, to all appearances, dead.

Subsequent events have been very clearly described by Shepherd[11]. A very fine outgrowth from the loop, at the point at which the nematode is attached, grows through the integument of the animal into its body. Once

inside, it expands to form the infection bulb, a globose structure that is seldom less than one-half the diameter of the eelworm, and which may be equal in diameter to the animal. From the infection bulb hyphae grow out into the body of the eelworm; these are the trophic hyphae by means of which the fungus absorbs the contents of its victim's body; they are septate, and about equal in diameter to the hyphae of the mycelium, or rather less. The trophic hyphae eventually fill the carcass of the captured eelworm. While they are growing, decomposition of the body contents of the animal is proceeding, and it becomes difficult to see the prorgess of the trophic hyphae because of the mass of oily decomposition products that is formed. Within 24 hours, however, the whole content of the eelworm is absorbed by the trophic hyphae, and is, one presumes, passed back to the mycelium. The final stage is the emptying of the trophic hyphae themselves. All that is then left is the integument of the eelworm, still attached to the network, and filled with empty hyphae.

In a petri dish culture containing a dense population of eelworms the slaughter caused by *Arthrobotrys oligospora* can be catacylsmic. Heaps of dead and dying eelworms obscure the networks that hold them, and are easily visible to the naked eye, while the smell of putrefaction is obvious to the nose as soon as the lid of the plate is lifted. Under the low power of the microscope the scene of carnage is almost harrowing.

Arthrobotrys oligospora is not always as active as this. Often it browses quietly on eelworms, catching a few here and there, but without apparently making any considerable inroads on the eelworm population of the culture. Why its behaviour should vary so much we do not know: that is one of the many points on which further information is urgently needed, for it may have an important bearing on the possibility of using predacious fungi for the biological control of plant pathogenic eelworms in the field. More will be said about this later on.

The reproduction of *Arthrobotrys oligospora*, as in other Hyphomycetes, is brought about by means of asexual spores (conidia). Erect fertile hyphae grow up vertically from the mycelium, and at their ends groups of colourless, pear-shaped, two-celled spores are formed (*Figure 4.1*). The spores are large, usually measuring 22 to 32 μ in length and 12 to 20 μ in diameter at the widest point. Of their two cells, the distal one is characteristically the larger. When the fungus is grown in pure culture, without eelworms, the spores are usually somewhat smaller. When a 'head' of spores has been formed at the end of the fertile hypha, others are produced lower down. These are carried in a series of whorls down the fertile hypha: there may be as many as 20 of these whorls, separated by internodes. This nodal formation of spores is more marked in pure cultures (where spore-production is usually very prolific) than it is in cultures containing eelworms.

Spores falling in suitable surroundings can germinate and so produce a new mycelium. Most commonly the germ tube grows out of the smaller basal cell of the spore. Nothing is known about the method of spore dispersal of this fungus. The spores are large, but not too large to be carried by air currents, though there is no positive evidence of this happening. The mites that, unfortunately, abound in cultures of predacious fungi can often be seen carrying the large spores of *Arthrobotrys* and other genera entangled with the

hairs on their bodies, but whether this is of any importance in Nature we can only guess. In any case, we know nothing about the sporulation of *Arthrobotrys* under natural conditions, and some knowledge of this must precede any useful attempt to theorize about the dispersal of the spores.

Since *Arthrobotrys oligospora* has large spores conveniently carried to a height of 0·5 mm or so above the surface on which it is growing, it is easy to isolate the fungus into pure culture by picking a spore off the end of a fertile hypha by means of a sterile glass needle and transferring it to the surface of sterile agar in a petri dish. The spores germinate well, and the fungus grows readily in pure culture, showing no sign of pining for a diet of eelworms. It forms a dense growth of mycelium, especially on one of the richer culture media such as potato dextrose agar, and produces plenty of spores. It does not, however, form any networks. *Arthrobotrys oligospora* in pure culture is, to all appearances, a normal hyphomycete: it shows no sign at all that it is a predacious fungus. This is true in general of the nematode-trapping Hyphomycetes.

If living nematodes are added to a pure culture of *Arthrobotrys oligospora*, networks begin to form and some of the eelworms are captured. It is not even necessary to add actual nematodes: a little sterile water in which eelworms have been living is enough to initiate trap formation. It seems that the formation of networks is induced by some chemical substance produced by nematodes. This is again generally true for the eelworm-trapping Hyphomycetes: they will form traps in pure culture in response to a chemical stimulus, and this stimulus can be provided by nematodes or by water in which nematodes have lived. The Hyphomycetes that capture eelworms with constricting rings are particularly interesting here, for, as we shall see presently, they will produce rings in pure culture in response to the presence of various substances of animal origin, such as serum or aqueous extracts of certain tissues.

The number of species capturing nematodes by adhesive branches is not large. The commonest is *Dactylella cionopaga* Drechsler, which has been recorded from Europe[12] and America[13]. The mycelium consists of septate hyphae, usually 3 to 4 μ in diameter. Short lateral branches grow out from the mycelium: these usually consist of one, two or three cells (*Figure 4.2*). Eelworms are captured by a sticky secretion produced by the branches, which function in the same way as the sticky networks of *Arthrobotrys oligospora*. When a captured nematode has become moribund, an infection bulb is intruded into its body, and trophic hyphae grow out of the bulb and absorb the body-contents of the victim.

The sticky branches of *Dactyella cionopaga* often proliferate, forming hyphal loops; sometimes several loops may occur together, but the complex three-dimensional networks found in *Arthrobotrys oligospora* are never formed. In spite of their relative simplicity, however, the eelworm traps of *Dactylella cionopaga* are remarkably effective.

Dactyella cionopaga reproduces by means of spores borne on the ends of erect fertile hyphae. The spores are large, ellipsoidal in shape, and usually consist of five cells, of which the central one is much larger than the others. They are formed singly at the ends of the fertile hyphae, but sometimes a fertile hypha may branch, producing a spore at the end of each branch.

The production of single multicellular spores is characteristic of the genus *Dactylella*, just as the formation of heads of two-celled spores characterizes *Arthrobotrys*.

The best known of the fungi capturing eelworms with sticky knobs is *Dactyella ellipsospora* (Preuss) Grove. This very common species has been recorded from four continents. The hyphae composing the mycelium are

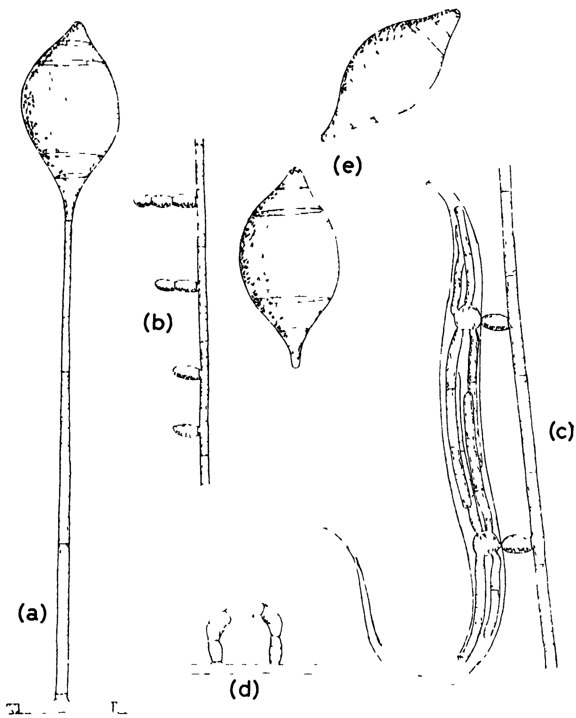

Figure 4.2. Dactylella cionopaga
(*a*) erect fertile hypa with a single terminal spore; (*b*) portion of mycelium with short adhesive branches, (*c*) eelworm captured by a pair of adhesive branches, showing infection bulbs and trophic hyphae; (*d*) adhesive branch proliferated to form a loop; (*e*) spores

rather slender, usually 2 to 3 μ wide. They are regularly septate. They produce at intervals short, two-celled branches, each of which carries a small, subspherical knob (*Figure 4.3*). The knobs are sticky and capture eelworms by adhesion in the usual way. Capture is followed by the formation of an infection bulb in the body of the eelworm, which then becomes filled with trophic hyphae.

The reproduction of *Dactylella ellipsospora* is similar to that of *D. cionopaga*. Spores are formed singly at the tips of erect fertile hyphae, which occasionally branch and produce a second spore. The spores are elliposidal and usually composed of five cells, the central one being the largest.

Some strains of *Dactylella ellipsospora* have a remarkable habit of proliferation from their adhesive knobs. Branches grow out from the knobs themselves and, curling round, anastomose with other similar branches, or with the parent hypha, thus forming loops. In this way, as in *D. cionopaga*, simple networks are formed. This habit seems to be much more marked in some strains of the fungus than it is in others, and is not at all uncommon.

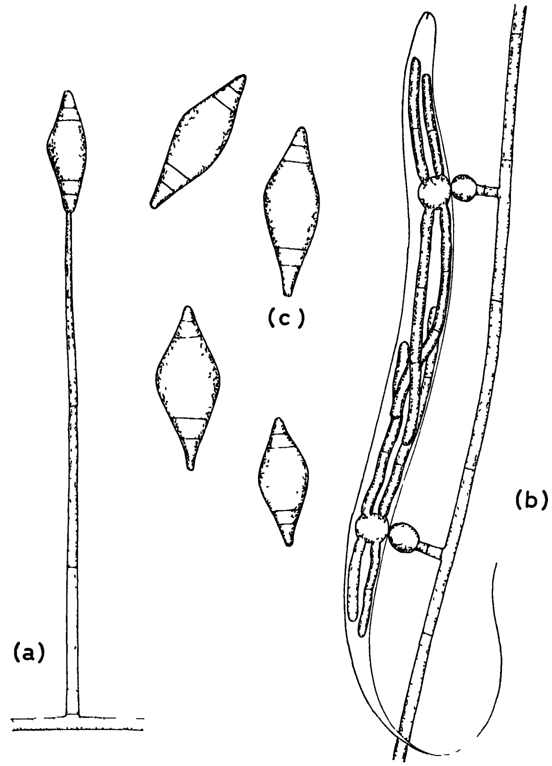

Figure 4.3. Dactylella ellipsospora
(*a*) erect fertile hypha bearing a single terminal spore, (*b*) eelworm captured by two adhesive stalked knobs, showing infection bulbs and trophic hyphae, (*c*) spores

The capacity for proliferation of the adhesive knobs appears to be genetically determined, for it persists through a number of generations of subcultures, and does not appear to be affected to any large extent by external conditions.

The mechanical traps of the predacious Hyphomycetes are of two kinds: non-constricting rings and constricting rings, according to whether the prey is caught passively by becoming wedged into the rings, or actively by being gripped by the expansion of the ring cells. A well-known species with non-constricting rings is *Dactylaria candida* (Nees) Sacc. This has a rather more delicate mycelium than most of the Hyphomycetes that prey on eelworms,

the hyphae being commonly about 1·5 to 3 μ in diameter. The mycelium bears stalked knobs similar to those of *Dactylella ellipsospora*, but their stalks are longer and more slender. In addition to the stalked knobs, the mycelium is provided with non-constricting rings.

The rings are formed from lateral branches of the mycelium. A branch grows out and then curls round on itself, the tip anastomosing with the

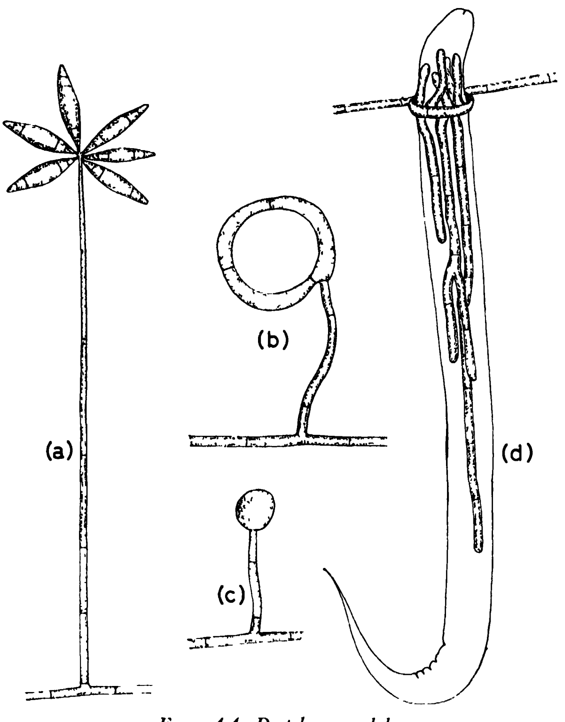

Figure 4.4. Dactylaria candida
(*a*) erect fertile hypha bearing a 'head' of spores; (*b*) non-constricting ring; (*c*) stalked adhesive knob; (*d*) eelworm captured by a non-constricting ring

lower part of the branch. In this way a stalked ring is formed (*Figure 4.4*). The ring usually consists of three curved cells, and the stalk is also usually three-celled.

The stalked knobs of *Dactylaria candida* capture eelworms in the same way as those of *Dactylella ellipsospora*, but their action appears to be rather feeble: small eelworms only are caught, and most of the predacious activity seems to depend on the rings. It is the rule rather than the exception for predacious fungi with non-constricting rings to be provided with stalked knobs, as well and it is also usual for the knobs to be secondary in action to the rings: in some cases they appear to be functionless.

The non-constricting rings are passive in their action: peripatetic eelworms that accidentally enter the rings do not have the wit to withdraw, and

become firmly wedged in their efforts to bullock their way through. There is no evidence that the ring cells are sticky, nor do they change their shape in any way when an eelworm has been captured. When an eelworm is held by one of the rings, there is the usual outburst of hysterical struggling. The struggles gradually become feebler and feebler, and, when the victim is finally dead or moribund, trophic hyphae invade its body from the ring and absorb its tissues.

The stalks that attach the rings to the mycelium are not robust, and it is possible for a captured eelworm, in the course of its struggling, to tear itself free and go on its way, wearing the ring like a collar. Its relief is, however, only temporary, for the rings appear to be able to function just as well when torn from the mycelium as they do when still attached to it. The eelworm is doomed: in due course, its body will be invaded by trophic hyphae from the ring in the usual way. The fungus does not stop at this, however. When the trophic hyphae have developed, a new mycelium grows out from the body of the victim. In this way the rings are able to serve as a means of vegetative reproduction as well as being organs of capture.

The spores of *Dactylaria candida* are, as in all the predacious Hyphomycetes, formed at the tips of erect fertile hyphae arising from the mycelium. The spores are fusoid in shape, and consist of from three to seven cells; they are formed in 'heads' of from three to 15 or more. The production of multicellular spores in heads at the tips of the fertile hyphae is characteristic of the genus *Dactylaria*.

We come now to the most remarkable of the eelworm traps used by the predacious Hyphomycetes—the constricting ring. This has been compared both with a rabbit snare and with a cowboy's lasso, although neither comparison is a very close one, as the ring works by the expansion of its component cells, and not by means of a running noose. *Dactylaria gracilis*[14] is a typical example of a hyphomycete with constricting rings.

The rings of *Dactylaria gracilis* are formed in a similar way to the passive rings of *D. candida*: lateral branches from the mycelium curl round and join up with themselves, forming stalked rings by anastomosis. In common with the constricting rings of other species, however, the stalk in this case is shorter, consisting of only two cells, and somewhat stouter. A captured eelworm does not easily tear itself free from a fungus with constricting rings: in fact, I have never personally known this to happen. The added stoutness of the stalk may be partly responsible for this, but no doubt the injury inflicted on the eelworm when the ring closes about its body is an important factor.

The constricting ring of *Dactylaria gracilis* consists of three cells (*Figure 4.5*) one of which is attached to the two-celled stalk. Each cell is curved, and thicker in the middle than at the ends: the result of this is that the ring is roughly circular in outline, with a three-sided opening. In petri dish cultures on agar the mycelium is mainly on or near the surface of the medium, and the rings are usually held perpendicularly on their stalks, like rabbit snares. This position makes it easy for an eelworm moving on the surface of the agar to slip its anterior end into the ring. A few seconds after this happens, the three cells of the ring suddenly swell to about three times their original volume: as the swelling is in an inward direction, the opening of the ring is occluded, with the result that the body of the eelworm is not

only firmly gripped, but severely constricted by the pressure of the ring cells. Closure of the ring is followed by the usual struggles on the part of the eelworm. These become feebler and feebler until finally they cease altogether When the eelworm is dead or moribund, trophic hyphae grow from the ring into its body and absorb its contents.

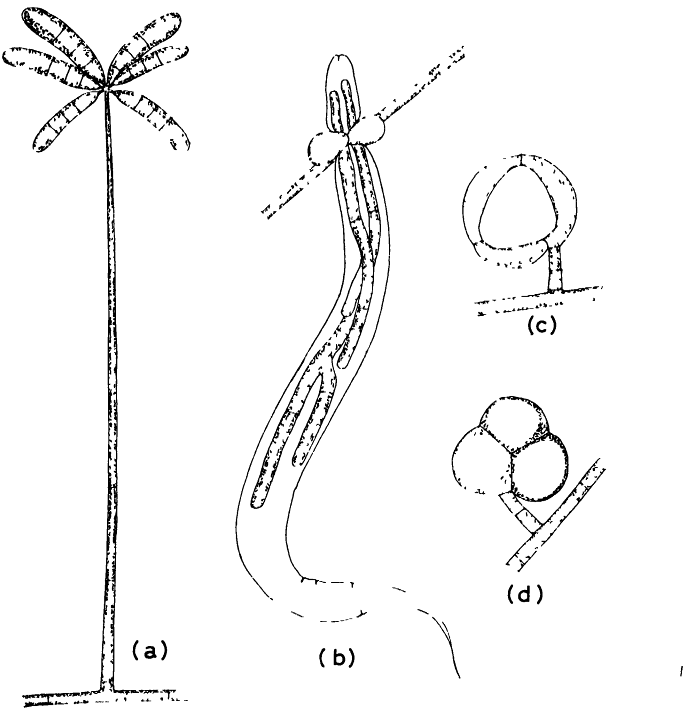

Figure 4.5. Dactylaria gracilis
(*a*) erect fertile hypha with a terminal 'head' of spores, (*b*) eelworm captured by a constricting ring; (*c*) ring in 'open' condition, (*d*) ring closed

Dactylaria gracilis reproduces by means of spores which, like those of *D. candida*, are formed in heads at the apices of erect fertile hyphae that rise up from the mycelium. The spores of *D. gracilis* are long and slender, and usually slightly curved: they consist usually of four or five cells.

We have now surveyed the more important methods used by the predacious Hyphomycetes in trapping eelworms. In all of these some special modification of the mycelium is present: even in those species that capture their prey by adhesion, the mycelium itself is not sticky, but only the traps. Sticky hyphae, however, may be found in the Zoopagales, where specialized traps are not normally formed and the prey is captured by adhesion to the vegetative hyphae of the mycelium.

The Zoopagales are far removed taxonomically from the Hyphomycetes. They belong to the Phycomycetes, the most primitive class of the Fungi, in

which the hyphae are not usually divided by cross-septa into cells, though septa are formed to delimit the reproductive structures and to cut off old, dead portions of the mycelium.

There are several species of Zoopagales that capture nematodes. One of these is *Stylopage grandis*. This fungus has a mycelium of rather stout, non-septate hyphae (*Figure 4.6*). Eelworms that touch the mycelium are caught

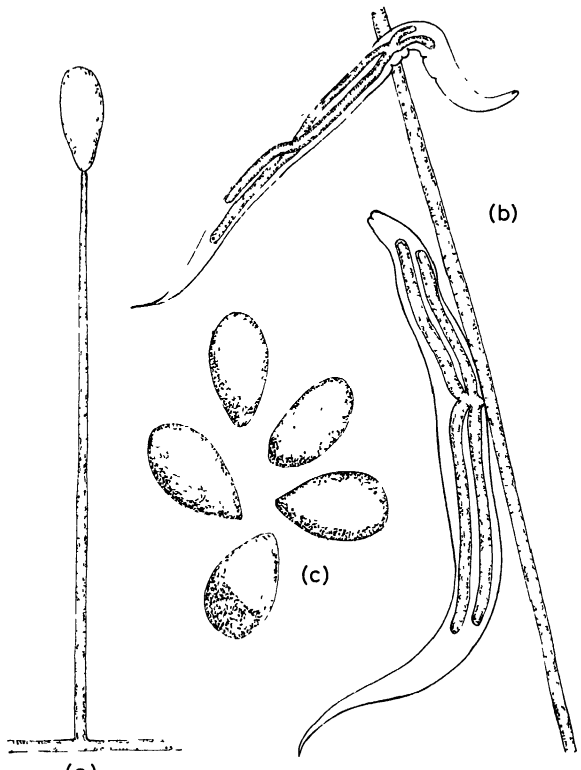

Figure 4.6. Stylopage grandis
(*a*) erect fertile hypha with a single terminal spore, (*b*) two eelworms caught by the sticky mycelium, (*c*) spores

by means of a sticky secretion; there are no specialized organs of capture, and there is nothing to suggest that any parts of the mycelium are stickier than the rest. When an eelworm has been captured, the glutinous fluid that holds it can clearly be seen as the captive attempts to tear itself away. Events follow the usual course: when the captive is moribund, trophic hyphae invade its body and absorb its contents. No infection bulb is formed in the body of the eelworm as there is always in the Hyphomycetes with sticky traps: the body of the eelworm is first penetrated by a short branch from the mycelium that holds it, and from this initial branch all the trophic hyphae

are derived. The trophic hyphae are a little smaller in diameter than the hyphae of the vegetative mycelium, and, like them, are without septa.

Stylopage grandis reproduces by means of large, ovoid or pear-shaped spores that are formed singly at the tips of tall, erect fertile hyphae: sometimes a fertile hypha branches and forms a spore at the tip of each branch. Many of the Zoopagales show sexual as well as asexual reproduction, but in *Stylopage grandis* this has not been observed.

All the nematode-trapping Hyphomycetes can be isolated in pure culture by the simple process of picking spores off the tips of the fertile hyphae with a sterile glass needle and allowing them to germinate on the surface of sterile agar in a petri dish. This is not the case with the Zoopagales: in fact, no predacious member of the Order has yet been isolated in pure culture. On a number of occasions I have tried to isolate *Stylopage grandis* by this method, as well as the eelworm-capturing *S. hadra* and the smaller *S. rhynchospora* that preys on amoebae. In no case have the spores germinated when transferred to sterile agar, though they do so freely when lying on the surface of a culture containing eelworms. It seems possible that these fungi, and possibly other members of the group, are obligate predators, unable to grow unless provided with their appropriate prey. This is in sharp contrast to the behaviour of the nematode-trapping Hyphomycetes, all of which grow freely in pure culture. It would seem as if the Zoopagales, morphologically unspecialized though they are, have attained a degree of physiological specialization that is not approached by the eelworm-trapping Hyphomycetes.

The absence of specialized organs of capture in the Zoopagales as a group may well be due to the fact that most of them prey on amoebae and other rhizopods with feeble powers of movement. It is reasonable to suppose that no great complexity of predatory apparatus is needed to hold an amoeba: in fact, the amoeba would probably aid its captor by clinging to the mycelium that held it. In this connexion, it is worth noting that *Dactyella tylopaga*, a predacious hyphomycete that captures amoebae, secures its prey by means of sticky hyphae alone, and without the aid of any definite organs of capture.

Nearly all the predacious Hyphomycetes prey upon eelworms, but there are a few species that take other prey. In addition to *Dactylella tylopaga*, *D. passalopaga*, *Tridentaria carnivora* and *Pedilospora dactylopaga* capture rhizopods, and it is perhaps not surprising that in these three species there is a notable lack of complex apparatus for capturing prey. It is noteworthy that the smaller Arthropoda, so common in Nature, seem to be passed over by the predacious fungi, though there is one species, *Arthrobotrys entomopaga*, that captures springtails (Collembola) by means of short branches of its mycelium with swollen adhesive tips. Probably even the smaller Arthropoda are too large and too active for predacious fungi to tackle: there is, after all no reason why they should hunt such relatively difficult prey with a copious supply of nematodes nearly always ready to hand.

The physiology of trap formation in the nematode-trapping Hyphomycetes has attracted the attention of several workers, but our knowledge of the conditions that lead to trap formation is still scanty. In general, they do not form their eelworm traps in the absence of nematodes, although, as we shall see in a moment, there are exceptions to this. They can be stimulated to form traps by the addition of nematodes, or of sterile water in which

nematodes have lived, indicating that the stimulus that initiates trap formation is a chemical one. What the active substance is, and whether there is one substance or whether there are several, we do not know.

The reticulate Hyphomycetes differ rather sharply from the species with constricting rings in their readiness to form traps in response to chemical stimulus. In general, the reticulate species produce their networks only in response to the presence of nematodes. There are exceptions to this: I have known a reticulate hyphomycete to start forming networks in eelworm-free cultures, apparently as a response to bacterial contamination, and I have also come across some strains of reticulate species that formed networks in abundance in pure culture for no obvious reason at all. It is still broadly true, however, that reticulate Hyphomycetes cannot be bamboozled into forming traps unless they are tempted with actual nematode exudations at least, if not with intact nematodes.

The Hyphomycetes that capture nematodes with constricting rings appear to be more willing to form their traps in the absence of eelworms, provided that they are given some sort of substitute. The first indications of this were obtained as early as 1937 by Couch[15], who observed that the ring-forming species *Dactylella bembicodes* would produce rings in pure culture on the addition of phosphoric acid, probably as a response to the lowered pH of the medium.

The question of ring formation in pure culture was taken up in France during the last war, and some very interesting observations were made. Roubaud and Deschiens[16] tried the effect of various substances of animal origin on pure cultures of *Dactylella bembicodes*. The solutions to be tested were poured on to pure cultures of the fungi growing in 10 cm petri dishes, and the number of rings formed, if any, was counted after a set time interval. This was the first attempt to obtain roughly quantitative information about the substances that were capable of stimulating ring formation. Roubaud and Deschiens counted the actual number of rings formed in a 10-cm petri dish, and scored their results as follows:

10–20 rings	+
20–50 rings	++
50–100 rings	+++
More than 100 rings	++++

They were thus able to compare the efficacy of various substances in initiating ring formation. Since they had observed that the presence of certain bacteria in the cultures could stimulate the fungus to form rings, they divided their test substances into those that were bacteriologically sterile, those in which only a few bacteria were present, and those which were heavily contaminated. They produced the following results:

I. *Sterile materials*

Human serum	++++
Human urine	+
Rabbit serum	+
Serum from patients infected by a parasitic worm	+++
Serum from a monkey infected by a parasitic worm	+++
Body fluid of *Parascaris equorum*, a nematode attacking horses	++++

II. *Materials containing few bacteria*	
Pulverized *Parascaris equorum*	++
Aqueous extract of a worm parasitic in monkeys	+
Aqueous extract of tapeworm	+++
III. *Materials heavily contaminated with bacteria*	
Aqueous extract of earthworms	++++
Pulverized mosquito larvae	++
Pulverized mosquito nymphs	+
Living mosquito nymphs	slight reaction
Pulverized fly pupae	+++
Extract of human dung	++++
Extract of monkey dung	+++
Extract of rabbit dung	+

Here we have an impressive list of substances, some of them entirely unconnected with nematodes, that can stimulate ring formation in *Dactylella bembicodes*. It will be noticed that human serum and dung extract were both highly active, while the serum and dung of the rabbit were feeble stimulants. Taken by itself, this would suggest that possibly a specific protein is concerned, but the idea is not supported by some of the other results. Both *Lumbricus* and *Parascaris* produced a strong reaction, yet one is an annelid and the other a nematode. The results show that ring formation can be stimulated by a wide variety of substances of animal origin, but they give us no clue to the chemical nature of the substance or substances that produce the effect.

Roubaud and Deschiens concentrated their attention on animal substances. A little later, Deschiens and Lamy[17] carried out similar experiments using plant products of various kinds, but in no case did they succeed in stimulating the formation of traps, although they observed that traps were sometimes produced in response to the contamination of their cultures with certain micro-organisms: these included *Euglena gracilis* and the yeast-like fungus *Torulopsis pararamosus*. These two organisms are taxonomically widely separated from one another.

Deschiens and Lamy also investigated the effect of guinea-pig serum on ring formation. They found that it was effective in dilutions as low as 1 part in 1,000 and that its activity survived both heating and treatment with alcohol. The stability of the active principle to heat is in direct contradiction to the work of Comandon and de Fonbrune[18], who found that the substance in water from eelworm cultures that stimulates trap formation loses its activity on boiling. This suggests that the active principle contained in guinea-pig serum may be different from the stimulating substance produced by living nematodes. These observations have not, however, been confirmed.

This work was continued by Lamy[19], who investigated quantitatively the effect on ring formation in *Dactylella bembicodes* of varying dilutions of the stimulant on mycelium of different ages. Using an aqueous extract of the earthworm *Lumbricus rubellus*, he tried the effect on: (*a*) young mycelium from germinating spores, and (*b*) older mycelium from agar cultures. Four dilutions of the crude earthworm extract were used: 1/10, 1/100, 1/1,000 and 1/10,000. The number of rings produced after 18, 25, and 42 hours'

immersion in the earthworm extract was counted, and the results are shown in *Table 4.1.* These results indicate that young mycelium produces traps more readily and more quickly than old, and also suggest that there may be an optimum concentration of the stimulating substance somewhere in the region covered by the 1/100 dilution of the crude earthworm extract.

Table 4.1

Dilution	*Age of mycelium*	*Response after:* 18 h	25 h	42 h
1/10	Young	+	++	++
1/10	Old	—	+	++
1/100	Young	+	+++	++
1/100	Old	-	+	++
1/1,000	Young	+	+	+
1/1,000	Old	—	+	+
1/10,000	Young	—	—	—
1/10,000	Old	--	—	—

These effects reappeared in further experiments on the same lines, using horse serum as a stimulant. Horse serum appeared to be less effective than earthworm extract in stimulating trap formation, higher concentrations being needed, but in general the results with horse serum echoed those obtained with the earthworm extract, as can be seen from the summary

Table 4.2

Dilution	*Age of mycelium*	*Response after:* 18 h	25 h	42 h
Undiluted	Young	—	—	
Undiluted	Old	-		—
1/2	Young	+	+++	++
1/2	Old	-	+	++
1/10	Young	+++	++	++
1/10	Old	+	+	+++
1/100	Young	+	++	++
1/100	Old	--	+	+++
1/1,000	Young	--	--	-
1/1,000	Old	—	—	-

shown in *Table 4.2.* With the horse serum the suggestion that there is an optimum concentration of stimulant is very striking, since undiluted horse serum is completely ineffective. On the other hand it is possible that the relative ineffectiveness of the higher concentrations of the stimulant may have been due to the presence of some substance or substances that inhibited trap formation when present above a certain concentration. This is a point worth attention in future investigations.

From a consideration of his experimental results Lamy drew five conclusions regarding the effect of earthworm extract and horse serum in stimulating trap formation in *Dactylella bembicodes:*

1. The extent of the reaction produced varies with the concentration of the stimulant.

2. With both earthworm extract and horse serum there is a lower threshold concentration below which no effect is produced.
3. At least with horse serum, there is an upper threshold concentration above which there is no effect.
4. For both stimulants there is an optimum concentration at which the greatest effect is produced.
5. Young mycelium reacts more quickly than old.

The fourth postulate is not valid on the evidence presented, since, as has been suggested, the presence of an inhibitory substance in both earthworm extract and horse serum could have produced the effects observed. However, there is no experimental evidence for the existence of any such inhibitor. The other four conclusions seem to be reasonably well established by the experimental data.

While ring-forming Hyphomycetes do not usually produce their constricting rings in any quantity without the presence of eelworms or some other appropriate stimulus, it must not be assumed that rings are *never* formed spontaneously in pure culture. In fact, most Hyphomycetes with constricting rings will produce a few traps without stimulation, especially on young mycelium, but the number of such traps is usually small.

Some recent work by Feder, Everard and Duddington[20] on constricting ring formation in *Dactylella doedycoides* suggests that this fungus is heterocaryotic with regard to the capacity to form rings easily. The cells of most Hyphomycetes are multinucleate, and it was shown by Hansen[21] that in a number of imperfect fungi two opposed strains could be obtained by making a large number of single-spore isolates. One strain, known as the M type, produced abundant mycelial growth, but sporulation was poor or absent. The other strain, called the C type, sporulated profusely but grew poorly. The normal form of the fungus, called the MC type, was a mixture of the two strains. Hansen called this the 'dual phenomenon', and postulated that a MC mycelium contained two types of nuclei, one determining a M type of growth and the other a C type. By making a large number of single-spore isolations, it should be possible to find a spore in which all the nuclei were of the same type: this, on germination, would give rise to a M or a C type of growth as the case might be. If a M type mycelium were mixed with a C type, anastomoses between hyphae would give rise to a normal MC mycelium, producing MC spores.

Something akin to Hansen's dual phenomenon seems to apply to ring formation in *Dactylella doedycoides*. In the work under discussion, the fungus was grown on corn-meal agar, and single spores were transferred, by means of a sterile glass needle, to the surfaces of corn-meal agar discs, each about 6 mm in diameter. These were kept at room temperature and the behaviour of the spores on germination was watched at intervals for 3 weeks. It was found that there were two distinct patterns of behaviour. The germling mycelium from some spores would produce from three to twelve rings soon after germination: spontaneous ring formation then ceased and was not resumed, even after 5 weeks. Spores of this kind were designated type A: they made up about 30 per cent of the total. Other spores produced germlings that bore no spontaneously-formed rings, even after 5 weeks' observation. These spores were designated type B.

Mycelia were grown from A and B type spores, and these in turn were tested in the same way. It was found that, in a large number of tests, 83 per cent of spores from A type mycelia produced rings spontaneously, while only 13 per cent of spores from B type mycelia did so. It thus appeared that the original isolate of *Dactylella deodycoides*, or wild-type, was separable into two distinct genetic races, differing in their capacity for spontaneous ring formation.

Tests were then made to see whether the two strains differed in their reactions to ring-forming stimuli. Saprophytic nematodes of the genera *Panagrellus* and *Rhabditis*, raised in laboratory cultures, were killed by air-drying and then rehydrated. Agar discs were sown with A, B and AB (wild-type) spores of *Dactylella doedycoides*, and a dead eelworm was placed on each of them. As a result of this stimulation, it was found that about 60 per cent of the wild-type (AB) spores produced rings within 3 days of germination. Of the spores from A type mycelium 87 per cent produced rings, while only 30 per cent of B type spores did so.

These results can be easily explained if we assume that the cells of *Dactylella doedicoides* are heterocaryotic with regard to the capacity for ring formation. It is assumed that in the A and B type mycelia there has been at least a partial sorting out of the two nuclear types: on mixing the A and B type myceliuma the AB (wild-type) is reconstituted. It would thus seem that ring formation in *D. doedicoides* shows a dual phenomenon akin to that found by Hansen. It would be interesting to test other predacious Hyphomycetes, and especially some of the reticulate species, to see whether this sort of dual phenomenon is general within the group.

The reticulate Hyphomycetes do not usually form their sticky networks in the absence of eelworms, and it is not easy to stimulate them to do so artificially. They will form networks if treated with sterile filtered water in which eelworms have lived, as was shown by Comandon and de Fonbrune[18], but, although the French workers did not state the concentration of the eelworms in the water they used, it seems likely that it must have been fairly high to get a response. I have tested *Arthrobotrys oligospora* and *A. robusta* with filtrates from cultures of *Ditylenchus dipsaci* containing 0, 6, 16, 64 and 256 nematodes/ml. respectively, with no result. In the same series of tests, the formation of adhesive branches by *Dactylella cionopaga* showed a slight but definite increase at the three higher nematode concentrations.

Some very interesting work on the stimulation of network formation in *Arthrobotrys conoides* by sterile filtrates of eelworm cultures has been carried out recently at Rutgers University, New Jersey, by Pramer and Stoll[22]. Their test eelworm was *Neoplectana glaseri*, which they grew in meat infusion broth supplemented with raw liver extract. Eelworm-free preparations were obtained by sterile filtration, and the crude filtrates were diluted as required with sterile distilled water. Various dilutions were made, and tested by adding 1 ml. of each dilution to the surfaces of 4-day-old plate cultures of *A. conoides;* the cultures were incubated at 28°C throughout the experiments.

The addition of an eelworm culture filtrate to cultures of *Arthrobotrys* conoides down to a dilution of 1 part in 100 was found to stimulate the fungus to produce traps: greater dilutions than this were found to be ineffective. A dilution of 1 part of filtrate in 5 parts of water was found to be the most

effective, confirming Lamy's observation that there is an optimum concentration of stimulant. In this respect, therefore, the adhesive networks of *Arthrobotrys conoides* behaved similarly to the constricting rings of *Dactylella bembicodes*.

Pramer and Stoll concluded that there must be a substance, given off by nematodes, that stimulates network formation. They called this substance 'nemin'. In subsequent experiments they assayed nematode culture filtrates for nemin by determining the greatest dilution at which it would stimulate trap formation: the reciprocal of this dilution they called the activity, expressed in dilution units. Thus, a filtrate that would produce traps when diluted 1/100 but not when diluted 1/200 would be said to contain 100 nemin units.

Next, Pramer and Stoll investigated the time at which nemin was produced by the eelworm *Neoplectana glaseri*, and also the stage in the life-history at which nemin production was greatest. Each of a series of tubes of culture medium was inoculated with 100 eelworms, and the tubes were incubated at 22°C, tubes being taken for testing after 4, 8, 12, 16, 21, 25 and 60 days. Each tube tested was freed from nematodes by filtering, a portion having first been taken in order to count the eelworm population: the filtrate was then assayed for nemin activity. It was found that only the 60-day cultures would stimulate trap formation: younger cultures were inactive. This seemed to indicate that the production of nemin was delayed until after the initial period of rapid multiplication of the nematodes, so that nemin did not appear in any quantity until the culture was aging. This is a point, however, that needs further investigation, for other explanations would seem to be possible: it might be that nemin only reached a high concentration in the medium after death and autolysis of large numbers of eelworms had set it free. The actual concentration of nemin required to initiate trap formation is not known: it is possible that several weeks are needed for the concentration of nemin in the culture medium to build up to a critical level.

Pramer and Stoll did not succeed in isolating nemin in a chemically pure condition, but they were able to gain some valuable information about its properties. They found that it was soluble in water, ethyl alcohol and n-butanol, but not in benzene, carbon disulphide or ethyl ether. It was not precipitated by acetone, and its activity withstood exposure to a temperature of 100°C for 10 minutes.

In order to concentrate nemin, a filtrate of a 4-month-old broth culture of *Neoplectana glaseri* was treated with four times its volume of acetone, and the resulting precipitate was removed by means of a centrifuge. The fluid portion was dried at room temperature, and the residue was dissolved in water. After extraction with n-butanol the butanol extract was dried at room temperature: the residue was then dissolved in water and tested for nemin activity. A similar extraction process was applied to human blood serum. Both extracts were active in inducing trap formation. It was considered that the method of extraction should have removed all protein, and also eliminated all polysaccharides of high molecular weight, so that it seems unlikely that nemin is one of the antigenic substances that nematodes are known to secrete. This work by Pramer and Stoll is exciting, and it is to be hoped that it will be followed up.

The reticulate Hyphomycetes are sometimes distinctly temperamental about network formation. Some years ago I isolated a reticulate species from garden compost, to which I gave the name *Trichothecium flagrans* on account of the high efficiency of its networks and the flamboyance with which it captured eelworms in enormous numbers. Within 3 months of being isolated in pure culture this fungus had entirely lost the ability to form networks, even in the presence of living nematodes, and it was no longer interested in eelworms. A few years later I was given another isolate of the same species that formed networks spontaneously and copiously in pure culture. I am unable to give any reason for this, any more than I can account for the fact that certain strains of reticulate Hyphomycetes will sometimes form networks spontaneously in pure culture for no apparent reason.

In general, predacious fungi do not lose their ability to form traps and capture eelworms through being kept in pure culture, even over considerable periods of time. Some of Drechsler's original isolates of predacious Hyphomycetes in the Baarn collection, tested recently by Juniper, showed no apparent loss of activity even after being deprived of eelworms for 20 years.

The formation of traps in the Hyphomycetes that capture eelworms by adhesive branches, and in those that employ sticky knobs, has received little attention by research workers. There seems to be a greater tendency towards the formation of traps spontaneously in pure culture than in the reticulate and the ring-forming species. *Dactylella cionopaga* commonly produces traps in pure culture, though the number of traps is greater in the presence of eelworms. *Dactylella ellipsospora* often forms a few traps in pure culture, but does so much more freely if supplied with eelworms: this has been noted by Comandon and de Fonbrune[18] and confirmed by other workers. Possibly this readiness to form sticky branches or knobs in pure culture may be correlated with the morphological simplicity of these structures compared with the much greater complexity of adhesive networks and constricting rings.

The working of the eelworm traps of the predacious Hyphomycetes has attracted the attentions of a number of research workers, from as far back as 1938, when Comandon and de Fonbrune[18] published their classical paper on the subject. The Frenchmen had recently designed a pneumatic micromanipulator of great delicacy, and they applied this to the investigation of the eelworm traps in *Arthrobotrys oligospora* (sticky networks), *Dactylella ellipsospora* (sticky knobs) and *Dactylaria brochopaga* (constricting rings), as well as to *Stylopaga hadra*, a nematode-capturing member of the Zoopagales, without specialized traps, in which the whole mycelium is sticky. The cinematograph film* that they produced in the course of their work is one of the most beautiful and exciting pieces of biological cinemicrography that has ever been carried out.

Comandon and de Fonbrune prepared microcultures of the fungi by sowing spores on thin cellophane membranes soaked in nutritive fluid. A piece of inoculated cellophane was spread out under a cover glass, and living, sterile nematodes were added. The sterile nematodes were obtained by

* Obtainable on hire from the Institut Français du Royanare-Uni, Queensberry Place, London, S.W 3.

treating nematode eggs from soil with 10 per cent eau de Javel: some hours later the eggs hatched and provided sterile larvae which could live for some days without bacterial contamination. It was found that 24 hours after the eelworms had been introduced into the fungal cultures, traps were formed, and that the number of traps became much greater after the first nematodes had been caught.

To handle the living eelworms under the microscope, glass micropipettes were used. These were made slightly narrower than the middle diameter of a nematode, so that an animal could be held by suction with its posterior end in the mouth of the pipette. In this way the eelworms could be pushed head first against the sticky traps, or their anterior ends could be introduced into the constricting rings.

Investigating the sticky networks of *Arthrobotrys oligospora* by these methods, Comandon and de Fonbrune found that the whole surface of the network was sticky. The networks were not equally sticky, and there seemed to be a correlation between stickiness and the age of the mycelium, the stickiness rising to a maximum and then declining with the age of the hyphae. Certain species of nematodes did not stick to traps that were highly adhesive for others: the traps were, to some extent, selective in their action. Under normal conditions, the traps were adhesive only for nematodes: they did not stick to other objects tested, such as a glass microneedle, chitin from insect cuticle, or the surface of small oligochaete worms, although in some cases some tardigrades were caught. If, however, the mycelium was left exposed to the air, it was found to stick to a glass needle, and a sticky substance which hardened rapidly was visible. The adhesive action of the networks was very rapid. Nematodes placed in contact with a network stuck fast in a few seconds, and could not be detached.

When a point on the cell wall of one of the cells composing a network was gently rubbed with the point of a microneedle the protoplasmic granules near that point began to move faster, and this excitation was communicated to the entire protoplasm of the cell, and sometimes to that of adjacent cells. At the same time, the granules began to move towards the point stimulated, where they collected round the edges of large hyaline vacuoles. Cells of the mycelium near the traps also showed this phenomenon, which also occurred in the sticky mycelium of *Stylopage hadra*.

The sticky knobs of *Dactylella ellipsospora* behaved similarly to the networks of *Arthrobotrys oligospora*. Nematodes placed in contact with them adhered quickly and firmly; adhesiveness reached a maximum at a certain period in the life of the mycelium; certain species of nematodes were not caught, and the knobs were adhesive only to nematodes.

In *Stylopage hadra* the whole surface of the mycelium was found to be sticky for certain species of nematodes, and here again adhesiveness reached a maximum at a certain stage in the development of a hypha, after which it gradually declined. The eelworms appeared to be fixed by a secretion, arising at the point of contact, which hardened rapidly: the existence of this secretion was pointed out by Drechsler when he originally described this fungus[23].

When the surface of a hypha of *Stylopage hadra* was touched with the tip of a microneedle a protoplasmic reaction similar to that observed in *Arthrobotrys*

oligospora was seen. In the mycelium of *S. hadra*, however, there are no cross-septa, and the protoplasmic excitement was transmitted to a considerable distance on either side of the point stimulated. It seemed probable that this accumulation of granular protoplasm was connected with the secretion of the sticky substance that held the nematode.

The close parallel between the action of the sticky traps of *Arthrobotrys oligospora* and *Dactylella ellipsospora* on the one hand, and the sticky mycelium of *Stylopage hadra* on the other, is particularly interesting when we remember the wide taxonomic separation of *S. hadra* from the predacious Hyphomycetes.

Investigating the action of constricting rings, Comandon and de Fonbrune worked mainly with *Dactylaria brochopaga*, but observations on the rings of *Dactylella bembicodes* suggested that the mode of operation was essentially similar in both fungi. When a nematode was held in a micropipette, and its anterior end was introduced into the lumen of a constricting ring, reaction followed quickly, the three cells of the ring swelling and gripping the nematode so firmly that the mycelium could be torn without freeing the worm.

It was found that the traps could be made to operate, without the presence of an eelworm, by mechanical stimulation. If the inside edge of one of the three cells of the ring was rubbed with a glass microneedle, it quickly swelled inwards, increasing to about three times its former volume, a figure since confirmed independently by Lawton and by Muller. In most cases the reaction of the stimulated cell was quickly followed by swelling of the other two cells of the ring, so that the microneedle was firmly gripped. The swelling process was very quick: measurements made by slow-motion cinematography indicated that the reaction time was of the order of one tenth of a second. The ring cells were only sensitive along their inner edges: stimulation of the outer edge of the ring with a microneedle produced no reaction.

The intracellular changes that took place when the rings closed were studied with the aid of slow-motion cinematograph films. The pictures clearly showed that, immediately after the swelling of the ring cells, a number of small vacuoles increased in size and finally ran together, forming, apparently, a single large vacuole. The significance of this was not apparent, but it clearly had something to do with the ring mechanism.

It was already known through the observations of Drechsler[10] that the non-constricting rings of *Dactylaria candida*, if torn from their moorings by the struggles of a captured eelworm, would still claim their prey, and that when this happened a new mycelium grew from the carcass of the victim. Comandon and de Fonbrune showed that the same was true of the constricting rings of *D. brochopaga*. A captured eelworm was removed, together with the ring that held it, and placed on sterile agar. A new mycelium soon grew from the cells of the ring, indicating that the constricting ring, like the non-constricting ring, can function as a means of vegetative reproduction as well as an organ of capture. It is unlikely that this is of much importance in Nature, however, as eelworms seldom, if ever, escape once they are held by constricting rings.

At about the same time as Comandon and de Fonbrune were engaged on their work, the action of the constricting rings of *Dactylella bembicodes* was

investigated by Couch[15], in the U. S. A. He agreed with the French workers that the closure of the rings was very rapid, and he observed that in old cultures the rings tended to lose their power to close. The vigorous way in which nematodes move suggested to Couch that the stimulus causing the traps to close might be a mechanical one, and he attempted to make the rings react by pushing the tip of a microdissector needle into the openings and moving it backwards and forwards in imitation of the movements of a nematode. No reaction followed.

This failure to secure mechanically-stimulated closure of the traps was directly contrary to the later findings of Comandon and de Fonbrune, and is a little difficult to understand. It is possible that the rings used by Couch were old and had lost their power of reaction, or it may have been that the movements of the microneedle were too gentle to evoke a response. It is unlikely that it was due to any peculiarity of the strain of *Dactylella bembicodes* that Couch was using, for he not only observed them operating normally against eelworms in culture, but also obtained artificial closure using other methods.

Couch next investigated the possibility that a chemical stimulus might be effective. He tried the effect of treating the rings with dilute solutions of ammonia, caustic potash, orthophosphoric acid, acetic acid, hydrochloric acid and sulphuric acid. The results of these tests were negative, although treatment with 1 per cent lactic acid caused a slight swelling of the ring cells.

Couch finally tried the effect of heat on the rings, and here he succeeded in making them close. Distilled water at any temperature between 33 and 75°C produced closure: below 30°C there was no response, while temperatures above 80°C killed the cells. Instead of using warm water, the rings could be closed by holding a hot scalpel near them. The discovery that the rings would respond to heat was a useful one, for it placed a powerful tool in the hands of future workers.

Ring cells that had expanded as a result of exposure to heat were seen to contain a large globule of a refractive substance, which Couch considered to be gelatinous or colloidal in nature. This suggested that the swelling might be due to some rearrangement of water and colloidal material already in the cell, or possibly the imbibition of water from the stalk, the speed of the reaction favouring the first hypothesis. More recent work[24], however, suggested that the globule may consist of water.

The operation of the constricting rings of *Arthrobotrys dactyloides* and *Dactylella doedycoides* has recently been investigated by Muller[24], using an ingenious technique by which the closing of the rings was slowed down by treatment with sugar solutions. Some of his observations are of considerable interest and shed new light on the details of this remarkable process. Muller found that the action of the rings could be slowed down about 100 times by treating them with concentrated sucrose solution, and then stimulating them with the same solution heated to 45°C before irrigating them with distilled water. In this way he was able to study the cytological events that occurred during closure directly under the microscope, instead of having to rely on information provided by a slow-motion cinematograph film. The improved resolution that resulted enabled him to make more detailed cytological

observations than had been possible with the technique used by Comandon and de Fonbrune.

Muller obtained constricting rings by immersing agar strips cut from cultures of *Dactylella doedicoides* in a 1 per cent solution of horse serum: he found that the 1 per cent dilution was more effective than the 10 per cent recommended by Lamy. The fungus, growing out from the sides of the agar strip into the solution, produced at the end of about a week a corona of mycelium well equipped with rings: this provided hyphae that could be stripped off and used for experiments.

Testing the effect of heat on the rings, Muller found that exposure to radiant heat from a hot scalpel produced constriction as did treatment of agar blocks bearing the fungus with hot water at temperatures between 31 and 85°C. Where tufts of mycelium were used instead of agar blocks, however, the maximum temperature producing constriction was found to be 54°C. Muller suggests, reasonably enough, that tufts of bare hyphae are more easily killed by heat than a mycelium growing on agar and therefore somewhat protected from the effect of the hot water, so that heat transfer is slower.

Muller found that, in the heat closure of the rings, shock treatment was necessary. When mycelium of *Arthrobotrys dactyloides* was gradually heated to 70°C on a slide, no closing of the rings occurred, nor could constriction be produced by subsequent treatment of the rings with hot water, probably because the rings had been killed by the previous heating. It was also found that the maximum temperature producing constriction varied with the age of the mycelium, being higher with 4-day-old rings than with rings that were 8 days old.

Muller also investigated the effect of other stimuli on the rings. He confirmed the observations of Comandon and de Fonbrune that closure could be produced by tickling the rings with a microneedle, the effect being the same with both *Dactylella doedycoides* and *Arthrobotrys dactyloides.* He tried the effect of intense light, firing a photographic flash bulb at a distance of only 5 cm from the rings: no closure occurred, and the treated rings subsequently reacted normally to hot water treatment. Exposure to ultraviolet radiation, although it did not cause the rings to close, inhibited completely their subsequent reaction to hot water. No constriction was produced when the mycelium, in a Buchner flask, was subjected to rapid changes of pressure with a filter pump, but it was found that some of the rings closed when a cover slip was dropped roughly on to a preparation. Passing electricity at 12 V d.c. and 240 V a.c. did not close the rings, but it was found that the rings exposed to 240 V a.c. were inactivated, since they did not close when later treated with hot water. Finally, the rings did not close when treated with hydrochloric acid, caustic soda, chloroform, ether, or benzene.

From these preliminary experiments, it became clear that heat treatment was the simplest and most reliable method of operating the rings artificially. In order to observe what happened when the rings closed, hyphae bearing rings were immersed in 0·3 to 0·5M sucrose solution for about 1 minute. The material was then removed from the sucrose and placed on a slide, with the smallest possible amount of liquid, and a drop of the same sucrose

solution, heated to 45°C, was allowed to fall on to it, after which the preparation was covered with a cover slip. The material was then irrigated with distilled water. Most of the rings closed on irrigation, and it was found that the rate of closure depended on the rate of irrigation: by careful adjustment it was possible to slow down the action of the ring by a factor of 100, the whole process of closing taking about 10 seconds instead of the normal 0·1 second. In this way, detailed observations of the cytological changes occurring during closure could be made under good conditions for microscopical observation.

Besides slowing down the ring action, it was possible by this method to obtain 'arrested stages' in ring closure by dropping very hot water on to the mycelium. The ring cells began to swell, but were apparently killed and fixed before the swelling was complete. A similar effect was produced by treating the rings with a 15 p.p.m. solution of neutral red for 15 minutes and then dropping water heated to 45°C on to them.

Working with *Dactylella doedycoides*, Muller found that the rings, in their normal or unstimulated condition, contained granular cytoplasm in which little or no Brownian movement was visible. The ring cells were seldom vacuolated, and the granular cytoplasm was evenly distributed. When a ring had been immersed in sucrose solution and then stimulated with sucrose solution heated to 45°C the granular material collected near the centre of the cell, leaving the peripheral cytoplasm almost clear. There was no plasmolysis.

When a ring in this stimulated condition was irrigated with distilled water, the granular cytoplasm in the centre of each cell produced a number of small vacuoles: these appeared to be without plasma membranes, at least at first, as indicated by the observation that cytoplasmic granules, in the course of Brownian movement, could pass from the granular cytoplasm into the vacuolar space and back again without hindrance, though this power of migration was lost at a later stage. The small vacuoles grew larger, and the coarser granules grouped themselves round them, producing what Muller termed the 'alveolar stage': this he regards as a stimulated pre-inflation stage.

The alveolar stage was followed by the stage of inflation. Here the sequence of events within the cells varied somewhat. In cells stimulated in 0·5M sucrose solution at 45°C the vacuoles increased in size and the inner cell wall bulged out into the lumen of the ring. As the vacuoles became larger some of them coalesced. At full inflation the cell contained a few large vacuoles instead of many small ones, and Brownian movement of the cytoplasmic granules, which had increased progressively during the process, became very vigorous.

In other cases, there was no growth of vacuoles during inflation. Rings stimulated in 0·3M sucrose at 48°C, for instance, showed an accumulation of water outside the general mass of granules, on the outer side of the cell, away from the lumen of the ring. Muller does not indicate whether this difference in behaviour was, in fact, a function of the conditions under which the rings were stimulated. In most cases, the final stage reached was a fully-inflated cell with a single large vacuole.

Muller studied the changes taking place in the cell walls during inflation

of the ring cells. The inner wall, bounding the lumen of the ring, became notably thinner as a result of inflation: in this Muller confirms the observations of Comandon and de Fonbrune. He also observed that in some instances there was a thickening of the inner cell walls during inflation at the junctions between adjacent cells, while in other cases there was an annular thickening at each end of the inflated cells. These thickenings were not universally present, however, and their appearance seemed to be subject to no obvious rule. The thickenings disappeared when inflation was complete. The inflation of the rings appeared to be irreversible, although if an inflated cell was plasmolysed there was a slight opening of the ring.

In experiments on the plasmolysis of both inflated and uninflated cells, Muller was able to show that there was no change in the osmotic potential of the cells following inflation. Testing uninflated rings with sucrose solutions of different osmotic pressures, plasmolysis of 50 per cent of the ring cells was obtained in solutions of 0·6M concentration. Ring cells of the same age, inflated by hot water treatment, showed 50 per cent plasmolysis at the same concentration of sucrose. It seemed, therefore, that inflation did not alter the osmotic potentials of the cells.

These observations of the osmotic potentials of ring cells are important. As Muller points out, if a sudden threefold increase in cell volume makes virtually no difference to the osmotic potential of the cell, then there must be a threefold increase of osmotically active material in the cell. The logic of this seems inescapable.

The increase in the extent of Brownian movement of solid particles in the cell during inflation is also interesting. This is taken by Muller to indicate the formation of regions of lowered viscosity during inflation, and again this seems to be a reasonable explanation. The evidence certainly suggests that viscosity changes come into the picture somewhere.

Muller's observations indicate that the plasma membranes are not damaged as a result of inflation—the fact that inflated cells can be plasmolysed alone would make this a necessary condition. Moreover, when an inflated cell is plasmolysed, the expanded inner cell wall does not collapse to any significant extent: it appears to have become 'fixed' in its new condition. This, as Muller points out, suggests that plastic changes have taken place in the cell wall itself. Observations on plasmolysed rings indicated that the expanded cell was still elastic for a time after expansion, but that it gradually became 'set': it would appear that the change in the cell wall is a gradual one.

Muller suggests that the phenomena connected with the inflation of the rings might be explained osmotically in one of two ways: a sudden increase in the osmotic potential of the ring cells followed by intake of water, or a decrease in wall pressure and an increase in permeability resulting in an increase in suction force. He also points out that the first theory is inadequate to meet the facts. If it were true, it would mean an increase of osmotic potential in the ring cells from the equivalent of 0·6M sucrose to the equivalent of 1·8M sucrose: the effect of this increase should be counteracted by immersing the rings in 0·2M sucrose solution, but, in fact, a sucrose solution of this strength was insufficient to prevent the closure of the rings. We therefore seem to be left with the second hypothesis. A rapid change in the

cell wall on the inner side of the ring, accompanied by an increase in permeability, would produce an increase in the suction force of the ring cells, resulting in the intake of water from outside. The inner cell wall, having become more elastic, would bulge into the lumen of the ring. The intake of water would, of course, have lowered the osmotic potential inside the cell: this would return to its former figure, either quickly or slowly, by the hydrolysis of polymers within the cell.

This theory is a very attractive one. It explains all the experimental observations, including the somewhat irregular appearance of peculiar thickenings of the walls of the ring cells: these, as Muller suggests, may well be due to the plastic slip of wall material during the reorganization of the cell wall during expansion. It is also in agreement with the curious fact that the osmotic potential of the ring cells is not appreciably affected by a threefold increase in volume, for, if we assume that the critical concentration of osmotically active material in the cell is not affected by the events leading to inflation, it is reasonable that the osmotic potential of the cell should be restored to its previous value when inflation is completed.

We must, however, sound one note of warning. Muller's observations were made on cells in which the inflation process had been artificially slowed down 100 times by exposing them to conditions that were wholly unnatural. While there is nothing in either theory or observation to indicate that the phenomena observed by Muller were in any way different from what happens in Nature, complete acceptance of an osmotic theory of ring inflation must await further work. Meanwhile, if direct evidence of the change in the nature of the cell wall of the ring before inflation could be obtained, Muller's hypothesis would be very difficult indeed to refute.

Very little work has been done on the electrical stimulation of constructing rings. Muller, as we have seen, failed to obtain closure of the rings with the application of either 12 V d.c. or 240 V a.c., but Te Winkel found that closure could be brought about by the use of a pair of microelectrodes connected to an induction coil. The response of the rings to a high-voltage current may well have been another instance of the 'shock-closure' observed by Muller when he dropped a cover glass on to a preparation of ring-bearing mycelium: on the other hand, it is possible that exposure to high voltages might affect both the permeability of the cells and the composition of the cell wall. This isolated observation by Te Winkel does not at the moment fit into an existing theory of ring closure.

THE ENDOZOIC PARASITES OF EELWORMS

The predacious fungi that are internally parasitic in nematodes differ sharply from the eelworm-trapping predators, and the two groups are not at all closely related taxonomically. All the major groups of fungi contain genera that parasitize nematodes: thus, we have *Nematoctonus*, an imperfect Basidiomycete, *Harposporium* and others that are probably imperfect Ascomycetes, *Meristacrum* among the Entomophthorales, *Euryancale* of the Zoopagales, *Protascus*, *Myzocytium* and others in the Lagendidiales and many others. A species of *Pythium* has been described as attacking the vinegar eelworm, and

I have seen free-living nematodes parasitized by an undescribed species of *Phytophthora*. The nematophagous habit is a very common one.

The best-known and the most widely distributed of the fungi endozoic in nematodes are found among the Hyphomycetes. *Harposporium anguillulae*, the first fungus to be described as predacious on nematodes, is one of the commonest of these: it has been very well described by Karling[25]. *H. anguillulae* has been found in a great variety of habitats, for instance soil,

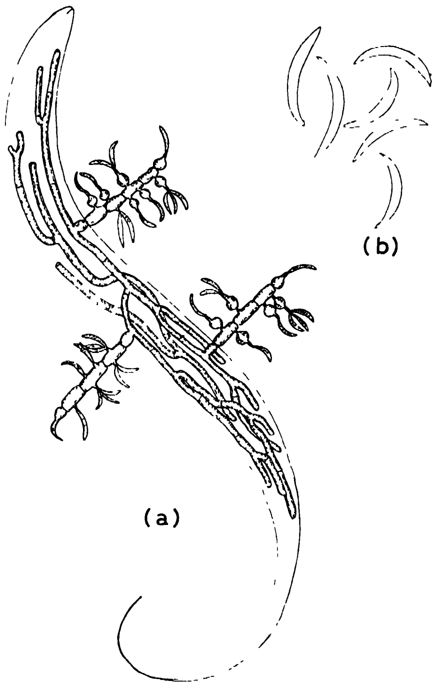

Figure 4.7 Harposporium anguillulae
(*a*) carcass of eelworm with internal mycelium and three fertile hyphae with sickle-shaped spores; (*b*) spores, in outline

dung, leaf-mould, rotting vegetation, moss cushions. It attacks nematodes through the agency of its spores, which are sickle-shaped and pointed at the ends. They are quite small, measuring usually 6 to 13 μ in a straight line from tip to tip (*Figure 4.7*).

There has been much uncertainty regarding the matter in which the spores of *Harposporium anguillulae* initiate infection of a nematode. Until recently it was assumed that they stick to the integument of the animal, either because one end of the spore is sticky, or by the pointed tip of the spore penetrating the skin. Recent work by Aschner[26], however, suggests that the spores may be swallowed, the arcuate shape of the spore helping to force it down the oesophagus of the eelworm as the animal moves its body from side to side. Aschner's evidence is impressive, but more work will be needed

before we can be certain that this is the sole method of infection, especially in view of the findings of other workers.

Entry into the host having been gained, germination of the spore gives rise to a mycelium of branched, septate hyphae within the body of the animal. At first the eelworm appears little the worse, but gradually its movements become sluggish and finally it dies, its body-contents being consumed by the growing mycelium, which eventually fills the carcass of the host.

The vegetative mycelium of *Harposporium anguillulae* is entirely within the body of the host. The reproductive phase of the fungus begins by the growth of fertile hyphae out through the host integument into the air. The fertile hyphae are usually no more than 40 μ long. They bear at intervals globular spore-bearing cells (phialides), each of which has, at its distal end, a short, narrow neck at the tip of which a group of about four sickle-shaped spores is formed. At maturity the spores become detached from the phialides and fall to the surface of the substratum, where they are readily picked up by eelworms, thus disseminating the fungus through the eelworm population. In a petri dish culture the slaughter caused by *H. anguillulae* can be considerable.

Very often the mycelium of *Harposporium anguillulae* forms chlamydospores inside, or sometimes outside, the body of the host. These are cells of the mycelium which become thick-walled, with dense cytoplasm, and which function as resting spores, suitable for tiding the fungus over a period of unfavourable conditions. *H. anguillulae* has considerable powers of survival: it can, for instance, readily be freeze-dried within the body of its host, and in this state will remain viable for long periods[27].

Several species of *Harposporium* have been described by Drechsler and others: all are parasitic in nematodes. *H. oxycoracum* and *H. helicoides* both have long, slender, strongly-curved spores with droplets of mucus at their ends which perhaps assist them in sticking to the integuments of nematodes. *H. subuliforme* has very short spores, each provided with a sticky spur. In *H. bysmatosporium* the spores are very small and shaped rather like the humerus bone of the human arm: they do not stick to eelworms, but are taken in through the mouth.

Another very common endozoic predacious fungus is *Acrostalagmus obovatus* which, like *Harposporium anguillulae*, attacks eelworms in a variety of different habitats, including soil. The spores of *A. obovatus* are very small, and are produced in large numbers. A spore sticks to the surface of a nematode, and on germination the germ tube penetrates the integument of the animal and gives rise to a branched, septate mycelium within its body. This grows until it fills the body of the host, most of the contents of which are absorbed during the process: fertile hyphae bearing spores then grow out to the exterior (*Figure 4.8*).

The fertile hyphae of *Acrostalagmus obovatus* are long and straggling, and in cultures they have a tendency to lie on the surface of the medium. The spore-bearing structures (phialides) of *Acrostalagmus* are slender and flask-shaped: in most members of the genus they are formed in whorls along the fertile hyphae, but in *A. obovatus* they are mainly formed singly or in pairs. This may well be a result of the procumbent habit of the fertile hyphae of *A. obovatus*, for in other species the fertile hyphae are strongly ascending.

The phialides on the fertile hyphae produce usually about 20 spores, which cohere together in a group round the neck of the phialide. These slimy phialospores stick easily to the outer surface of an eelworm, and it is not unusual to see eelworms with numerous spores sticking to them, especially around their anterior ends.

Meria coniospora is also a common endozoic hyphomycete which attacks eelworms in a similar way to *Harposporium* and *Acrostalagmus*. Here the fertile

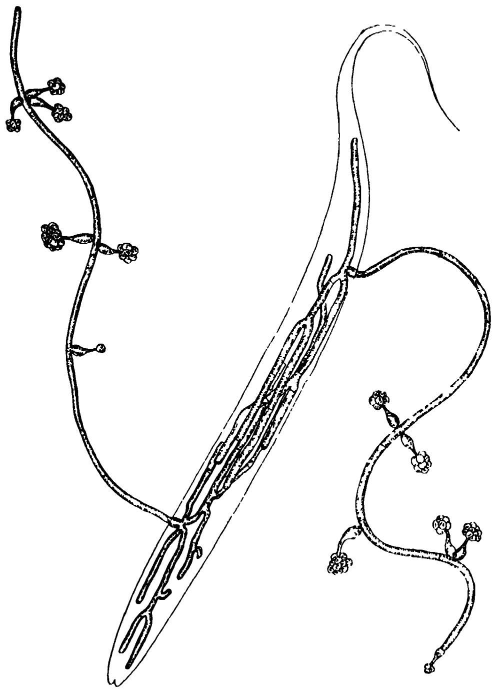

Figure 4.8. Acrostalagmus obovatus
Carcass of infected eelworm showing internal mycelium and two external fertile hyphae bearing flask-shaped phialides with groups of spores clustered round their necks

hyphae that emerge from the dead host are short and erect, bearing large numbers of triangular spores (*Figure 4.9*).

There are several species of *Nematoctonus* that attack nematodes. These have a more profuse habit of growth than any of the endozoic Hyphomycetes so far dealt with, and the hyphae are coarser. In *N. tylosporus*, for instance, the fertile hyphae that emerge from the body of the dead host often form a tangle on the surface of an agar culture. At intervals they bear, standing erect on short sterigmata, large club-shaped spores (*Figure 4.10*). At the distal end of the spore is a small protusion, which appears to be adhesive. The spores stick to eelworms and give rise to an endozoic mycelium in the usual way.

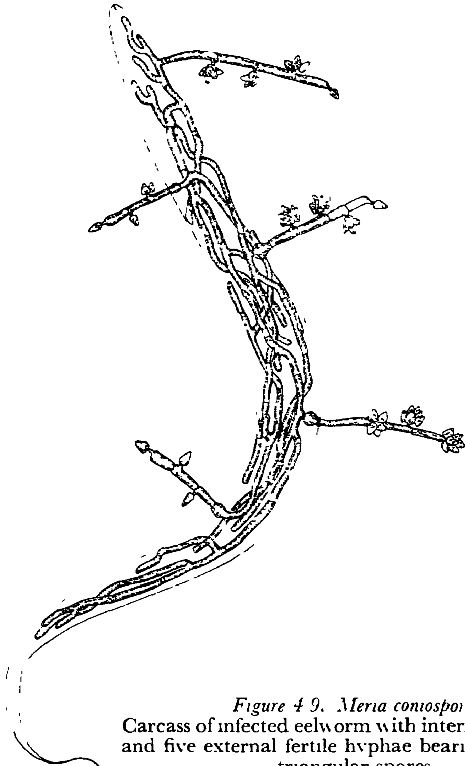

Figure 4 9. Meria coniospora
Carcass of infected eelworm with internal mycelium and five external fertile hyphae bearing clusters of triangular spores

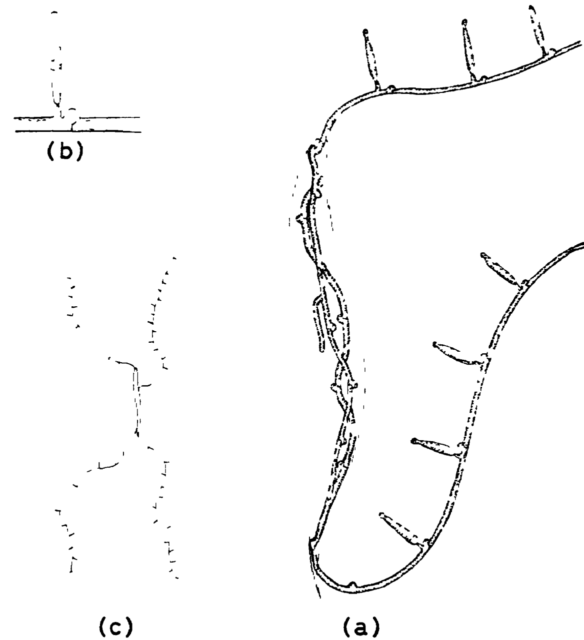

Figure 4.10. Nematoctonus tylosporus
(*a*) carcass of infected eelworm, showing internal mycelium and proximal ends of two fertile hyphae with clamp connexions and spores. (*b*) part of fertile hypha, with spore; (*c*) plan of infected nematode, showing length of fertile hyphae

The genus *Nematoctonus* is particularly interesting in two ways. In the first place, both the vegetative mycelium inside the host and the fertile hyphae outside bear clamp connexions, small outgrowths of the cell wall opposite each of the cross-septa, giving the appearance of a 'by-pass' round the septum. The presence of clamp connexions is characteristic of the Basidiomycetes: they are not found in any other group of fungi. This would seem to indicate that *Nematoctonus* is an imperfect Basidiomycete.

Nematoctonus is also interesting because some of its species bridge the gap between the nematode-capturing and the endozic predacious fungi. In *N. haptocladus* the fertile hyphae that emerge from the carcass of the host produce large, oval spores that stick to the bodies of nematodes in the usual way. The fertile hyphae also form, usually at their tips, dumb-bell shaped outgrowths that are sticky, and which function in much the same way as the sticky traps found in the nematode-trapping Hyphomycetes. Eelworms are captured on these outgrowths by adhesion, and their bodies are invaded by trophic hyphae that consume their contents. *N. haptocladus* is thus both an endozoic parasite and an eelworm-trapper. This tendency is even more strongly marked in *N. concurrens*, where the sticky branches are formed in large numbers all along the fertile hyphae.

Several interesting predacious fungi are to be found among the Lagenidiales, a primitive order of fungi, most of which are parasitic in fresh-water algae. At least two of these have been known for more than half a century.

Protascus subuliformis was first described as a parasite of eelworms as far back as 1903. This fungus has club-shaped spores, which are somewhat curved and pointed at one end. The spores stick by their pointed ends to the bodies of eelworms, sometimes in considerable numbers (*Figure 4.11*). On germination, a germ tube from the spore penetrates the integument of the eelworm, the protoplast from the spore entering the body of the host as a small, irregular mass of protoplasm. This increases in size, elongating as it does so, growing eventually into a broad, irregular filament which fills the greater part of the body of the host. Later the filament becomes divided by cross-septa into a number of portions that break apart from one another as the time for spore formation becomes near.

Spore formation is holocarpic: each of the thalli formed by the breaking-up of the original filament becomes a sporangium, and its contents divide up to form a large number of club-shaped spores. An exit tube grows out through the integument of the host to the exterior, and the spores are ejected. The mechanism by which the spores are forced out of the sporangium has not been elucidated: it has been suggested that the hydrolysis of some such substance as glycogen in the sporangium produces an increase in osmotic potential, shooting out the spores by the pressure of water taken in, but there is no evidence for this, and the sporangia appear to be practically devoid of residual cytoplasm after the spores have been delimited.

Besides the copious production of asexual spores, *Protascus* undergoes a form of sexual reproduction in which two adjacent thalli conjugate by the formation of a short conjugation tube between them, the contents of one thallus passing over into the other. As a result of this process a thick-walled resting spore is formed. As multiple infection of an eelworm is the rule rather than the exception, the remains of dead eelworms can often be seen

in cultures, occupied by a row of resting spores. When even the integument of the eelworm has disintegrated, the row of resting spores will be left to mark the spot where the body lay.

Protascus subuliformis differs from most other Lagenidiales in having non-motile spores, for the normal asexual reproduction in the group is by means if biflagellate zoospores. It seems very likely that this is an adaptation to parasitism in a highly mobile and gregarious host: eelworms exist in

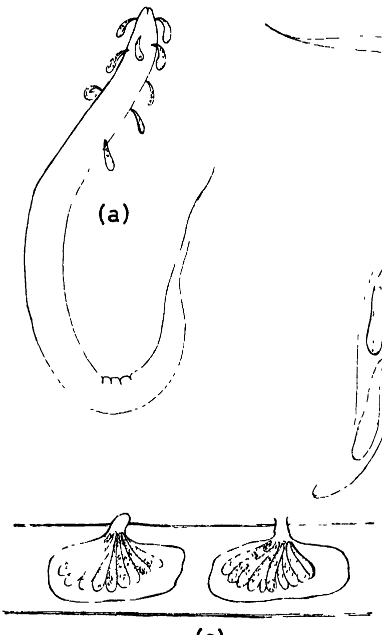

Figure 4.11. Protascus subuliformis
(*a*) eelworm with a number of club-shaped spores adhering to it, (*b*) carcass of eelworm containing several thalli; (*c*) two sporangia in the body of an eelworm, with spores ready for ejection

countless numbers, and they move about quickly and freely, so that the possession of motile spores by a fungus attacking them would be of little advantage, and might even be a disadvantage. In the circumstances, an adhesive spore would have obvious advantages over one that was merely motile. It is interesting that *Haploglossa heterospora*, another nematophagous member of the Lagenidiales, also has non-motile, sticky spores, and that in *Myzocytium vermicolum*, a nemaphagous member of the same group that normally produces zoospores, it is not uncommon for the spores to develop adhesive outgrowths instead of flagella.

Myzocytium vermicolum (*Figure 4.12*) is another predacious fungus that has been known for a long time, for it was first recorded, under a different name, in 1893. In its general features and life-history in resembles *Protascus*: thalli

formed within the host become sporangia at maturity and spores are liberated through an exit canal. Here, however, the spores are zoospores, in which respect *Myzocytium* resembles other members of the Lagenidiales. Very often, however, the spores fail to develop flagella: instead they produce small, bud-like outgrowths at one end, and these, by proliferation, may become short moniliform branches. The outgrowths are sticky and enable

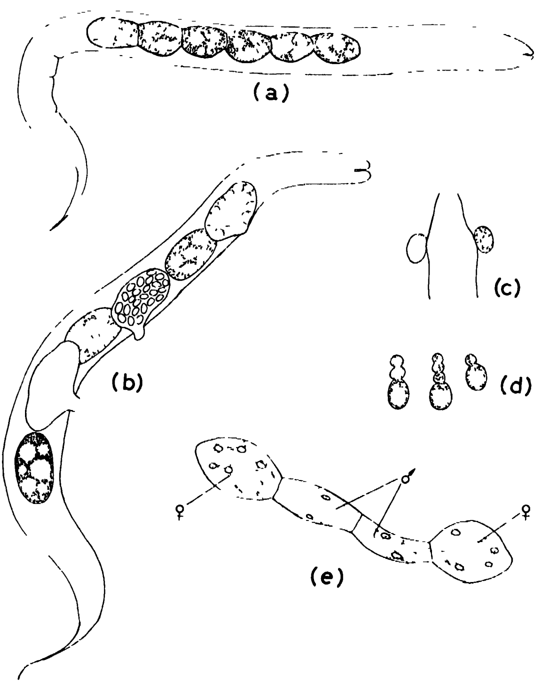

Figure 4.12. Myzocytium vermicolum
(*a*) body of an eelworm containing a six-celled thallus, (*b*) later stage: the thallus has broken up into its individual cells which are developing into sporangia, one of which has already discharged its spores; (*c*) zoospores; (*d*) aplanospores with moniliform adhesive buds, (*e*) sexual thallus with two terminal oogonia and two central antheridia (after Dangeard)

the spores to stick to nematodes with which they come into contact. *Myzocytium* appears to be slightly less specialized for predation than *Protascus.*

There are several other nematophagus members of the Lagenidiales These include *Haptoglossa heterospora,* a fungus that has been recorded from America, England and Denmark. Here again the spores are non-motile, as in *Protascus,* and each is provided with an irregular adhesive outgrowth. The original specimen of this fungus, described by Drechsler, was remarkable in having spores of two different sizes.

One of the strangest nematophagous members of the Lagenidiales is *Gonimochaete horridula,* described by Drechsler as attacking eelworms in

decaying maple leaves in America. The sporangia formed in the body of the eelworm have remarkably long exit tubes which stand out vertically from the carcass, giving it a spiky appearance. The spores are small, cylindrical and non-motile: they have sticky appendages by which they stick to eelworms. The taxonomic position of *Gonimochaete* is a little uncertain, but it is probably best placed in the Lagenidiales with *Protascus* and *Myzocytium*.

Euryancale sacciospora is interesting as a member of the Zoopagales that is endozoic in eelworms. This is unusual in the Zoopagales, though many of them are internally parasitic in Protozoa. *E. sacciispora* forms a non-septate mycelium in the bodies of eelworms, which it attacks by means of sticky spores.

PREDACIOUS FUNGI IN SOIL

The ecology of the predacious fungi has been very little studied, although it is a field that offers great opportunities to the research worker. Some of the predacious fungi that attack eelworms are very common, and they are to be found in almost any kind of habitat that will support a population of free-living nematodes. A species of *Pythium* was observed by Sadebeck attacking the vinegar eelworm in vats of vinegar, and I have found eelworm-trapping species in moss growing between city paving stones and in soil from the recently-exposed foundations of bombed buildings in the City of London. Predacious fungi have been recorded from soil, rotting wood, dung, vegetable compost and leaf-mould, moss cushions, rotting vegetation immersed in water and many other habitats. There is only one record of a marine predacious fungus[28], but we must remember that our total knowledge of marine fungi is small: further investigation in this field may well bring to light other predacious species from salt-water habitats.

One of the greatest difficulties in the study of the habitat relations of predacious fungi has been the lack of an adequate sampling technique. Their detection and identification has up to now depended on making petri dish cultures from small pieces of suitable material, which are themselves sub-samples of the environment. Moreover, there is as yet no known means by which one can be sure that even a small piece of material has yielded all the predacious fungi that it contains. A collection of a few hundred grammes of leaf-mould or rotting wood, if kept moist, may continue to produce different species when sampled over a period of months or even years. Another handicap to ecological studies has been the lack of any method of making quantitative studies of the occurrence of predacious fungi, or of their activity under different conditions and in different habitats. The agar disc technique recently devised by Cooke[29], of which more will be said later, may well be of inestimable value here.

The study of predacious fungi in soil is of particular interest because of the damage done to crops by plant parasitic nematodes. The fungi appear to be present in most fertile soils, and it seems likely that they prey on the free-living nematodes that abound almost everywhere. A survey of predacious fungi in English arable soils[30] produced 82 records of fungi attacking nematodes from 49 samples of soil: in all, 21 species were represented. Of these, the commonest was *Arthrobotrys oligospora*, which accounted for no less

than 21 out of the total of 82 records. Further observations since then have confirmed that *A. oligospora* is extremely common in soil, and it is interesting that Shepherd found that this species is equally common in Denmark: 70 samples of Danish agricultural soils yielded 62 records of nematode-attacking fungi belonging to 10 species, *A. oligospora* being recorded from no less than 35 of the 70 soil samples.

Although predacious fungi are widespread in soils of a wide variety of types, they seldom appear in published lists of fungi found in soil. This is probably because the methods used in isolating fungi from soil are unsuitable for revealing the presence of predacious species. It is a common practice in soil mycology to isolate soil fungi on media such as Czapek–Dox agar at pH 4·2 or thereabouts, the purpose of the low pH being to keep down the growth of bacteria. (In general fungi will grow, at least after a fashion, under more acid conditions that most bacteria will tolerate.) Such acid conditions would be quite unsuitable for most predacious fungi, and it is therefore hardly surprising that they are seldom found. This is a point to remember when evaluating work on soil fungi: where media of low pH or other extreme conditions are used in sampling the fungus flora of the soil the results are bound to be epistomological to some extent, a fact that is not always realized as fully as it might be.

The activity of predacious fungi in the soil under natural conditions is an important field of investigation, especially in view of their possible use for the biological control of eelworms. Do they, in fact, prey actively on the soil nematodes, of which there are usually plenty, or do they exist in the soil mainly as saprophytes, or as resting spores? Drechsler[10], and Linford and Yapp[31], have pointed out that the nematode-trapping Hyphomycetes do not compete well with moulds for the use of organic substrates in the absence of nematodes. If eelworms are present, however, the nematophagous habit gives the predacious fungi a biological advantage over their competitors, as can readily be seen in laboratory cultures. It is therefore logical to expect that in soil, where there is a thriving mould flora, the nematode-trappers will take advantage of their peculiar method of feeding, and there is both direct and indirect evidence that this is so.

The agar disc technique of Cooke[29] promises to be a potent tool in investigating the activity of predacious fungi in soil. Cooke cut circular discs of maizemeal agar, 1 cm in diameter and about 3 mm thick: the discs were placed on microscopic slides, which were then buried about 1 cm deep in soil in 15-cm petri dishes. Four discs were used on each slide, and the slides were removed from the soil at weekly intervals for examination. The soil was washed off the surface of the agar with a fine jet of water, leaving intact any fungi that had grown there. Using the discs as microquadrats, Cooke was able, not only to detect the presence of predacious fungi in his soil samples, but also to estimate their abundance and state of activity in capturing nematodes.

Cooke[32] estimated the activity of predacious fungi in two ways—by counting the number of different types of eelworm traps that appeared on the four discs on each slide, and, with the network-forming fungi, by counting the average number of adhesive loops per disc. He expressed his results in terms of a quantity that he called 'activity factor'. In this way he was able

to make a quantitative estimate of the effect of different soil treatments on the activity of the predacious fungi that were present. In particular, he investigated the effect of adding easily-decomposable plant material to the soil on the population of free-living nematodes and on the activity of predacious fungi, with remarkable results.

The fact that the predacious activity of nematode-trapping fungi is stimulated by the addition of green plant material to the soil was first demonstrated by Linford[33], in experiments on the biological control of the pineapple root-knot eelworm in Hawii. He found that the addition of chopped green pineapple tops to the soil produced a rapid increase in the population of free-living nematodes, and that this was followed by an increase in the activity of predacious fungi. It was assumed that the increased activity of the fungi was due to the stimulating effect of the rise in the eelworm population, giving the fungi more scope for their voracity. This hypothesis has been generally accepted for more than two decades, but in the light of observations made by Cooke it is no longer tenable.

Cooke plotted the activity of predacious fungi over a period of several weeks in soil to which chopped cabbage leaf tissue had been added: at the same time, regular estimations of the nematode population of the soil were made, using a modified Baermann funnel technique[34]. At the end of the first week both predacious activity and the nematode count began to rise sharply. Predacious activity reached a maximum in about 3 weeks, and then declined quickly, becoming negligible at the end of about 6 weeks. The nematode count reached a maximum a little later than the maximum for predacious activity and then slowly declined. At the end of 6 weeks, however, the nematode count was still very high, higher, in fact, than it was when predacious activity was at its greatest. This experiment was repeated several times, under carefully controlled conditions, with the same result.

In addition to using chopped cabbage leaf tissue as a stimulant for predacious fungi, Cooke tried the effect of adding sugars to the soil, with remarkable results. The addition of 1·2 per cent by weight of sucrose to the soil produced a startling rise in the activity factor, and this effect was maintained over a period of 10 weeks. The stimulating effect of sucrose on the fungi was much greater than the effect produced by adding chopped cabbage tissue, and the stimulation lasted for nearly twice as long. On the other hand, the effect of sucrose on the nematode population was much less than the effect produced by chopped cabbage. In one experiment the nematode population rose from an average of five nematodes/g of soil to a maximum of 112/g under sucrose treatment, while in the same experiment the addition of chopped cabbage to the soil produced a maximum nematode population of 297/g.

Cooke's experiments showed that both chopped cabbage tissue and sugar could act as stimulants to the predacious activity of soil predacious fungi, although their effects were quite different. Chopped cabbage produced a transient rise in fungal activity accompanied by a much more lasting rise in the nematode population: with the sugar these two effects were reversed. One effect was common to both treatments, however: there seemed to be no quantitative connexion between the activity of the fungi and the number of nematodes present in the soil.

It seems clear from these observations that we must reject Linford's hypothesis that the stimulating effect of organic matter on soil predacious fungi is a function of the rising nematode population. It looks as if a more direct effect of organic matter on the fungi must be sought. The solution of this interesting problem may have important results in the field of biological control of nematodes.

PREDACIOUS FUNGI AND NEMATODE CONTROL

The first experiments on the use of predacious fungi for the biological control of nematodes were made by Linford and his co-workers in Hawaii, shortly before the Second World War[31,33,35–37]. They were working on the pineapple root-knot eelworm, which at that time was causing considerable havoc in the Hawaiian pineapple fields. Attempts to control the root-knot eelworm by inoculating the soil with pure cultures of predacious fungi were not successful. Six isolates of nematode-trapping Hyphomycetes were used: *Arthrobotrys musiformis* (sticky networks), two isolates of *A. oligospora* (sticky networks), *Dactylaria thaumasia* (sticky networks), *Dactylella ellipsospora* (sticky knobs) and an inidentified species of *Dactylella*. The treated soil was contained in 5-gallon pots, and was artificially infested with eelworm by adding larvae of the root-knot eelworm. Pineapple plants were grown in the pots, and the experiment was kept going for 15 months. At the end of this period eelworm damage to the pineapple plants was assessed by measuring the top growth of the plants, and also by measuring the extent of the root system. In this experiment only *Dactylella ellipsospora*, out of the six isolates tested, produced any indication of reduction of eelworm damage when compared with the untreated control. The other five fungi were apparently without effect.

In place of soil inoculation, Linford and his colleagues next attempted to make use of the predacious fungi occurring naturally in the soil, a previous survey having shown that these were widely distributed in the Hawaiian pineapple fields. It seemed logical to suppose that if the naturally-occurring predacious fungi could be stimulated into enhanced activity, artificial inoculation of the soil might well be unnecessary. In this series of experiments, soil naturally infested with root-knot eelworm was used in 5-gallon jars as before. No inoculation with predacious fungi was given, but chopped green pineapple tops were incorporated into the soil. It was found that this treatment produced a rapid increase in the numbers of free-living eelworms in the soil: in one experiment the eelworm population rose to 65 times its former level within a fortnight of adding the green manure. The root-knot eelworm, being an obligate parasite, was not affected. This dramatic increase in the eelworm population was followed immediately by an equally rapid fall, the numbers of infective larvae of the root-knot eelworm falling along with the rest. During the period of falling eelworm population predacious fungi appeared to be extremely active in the soil.

Linford and his colleagues considered that the increase in the eelworm population that followed green manuring stimulated the predacious fungi in the soil into greater activity, and that the fungi then proceeded to clean up, not only the free-living eelworms that had stimulated them, but the root-knot eelworms as well. The net result, therefore, was a reduction in the

population of root-knot eelworms. While the work of Cooke makes it impossible to accept the idea that it is the rise in eelworm population that acts as a stimulus to the fungi, Linford's principle of fungal stimulation by green manuring appears to have been sound, and his observations have been amply confirmed by other workers[29,38,39].

Having established the effect of green manuring on eelworm population and fungal activity, Linford carried out a number of experiments on the control of root-knot eelworm. Soil was collected from land heavily infested with root-knot eelworm and placed in 1-gallon glass jars, weighed quantities of the organic amendment being added to the jars and mixed with the soil. During the period of decomposition of the organic materials the mouths of the jars were covered with muslin, and the jars were weighed at intervals, any loss in weight being made up by adding water. After the decomposition of the organic material had proceeded for a given time, the soil from each jar was well mixed and then distributed between four flower pots, in which seeds of the cowpea (*Vigna sinensis*), a plant susceptible to pineapple root-knot eelworm, were sown. About 5 weeks after sowing, the cowpea seedlings were removed from the pots, their roots were washed, and the number of eelworm galls on them was counted.

In the first experiment along these lines, three different organic amendments were tried: chopped pineapple tops, chopped leaves of the coarse grass *Panicum barbinode*, and sugar. Decomposition was allowed to proceed for 12 weeks, after which the soil was transferred to pots in which cowpea seeds were sown. Samples of soil from the jars were found to have a higher eelworm population where an organic amendment had been used than in the controls. Examination of the soil for predacious fungi disclosed the presence of four different species in the pineapple series, three in the grass series, and none in the sugar series or in the controls. Cowpea seedlings grown in the pots to which the soil was transferred showed marked differences in eelworm damage according to the several treatments. The seedlings from the soil treated with pineapple or grass had fewer galls on their roots than those from the control soil: the seedlings from the soil that received sugar unfortunately failed to grow. After removal of the cowpeas, pineapple plants were grown in the pots. After 10 months' growth it was found that those grown in the soils that had been treated with organic matter were greatly superior to those from the control pots, both in top growth and in extent and condition of the root system.

It will be noted that Linford failed to observe activity of predacious fungi in soil treated with sugar. His sugar dosage, however, was rather heavy (over 4 per cent by weight), so that the inactivity of predacious fungi was hardly surprising. Cooke found that sugar stimulated the activity of predacious fungi at a concentration of 1·2 per cent by weight, but that the stimulating effect was very slight at 2 per cent, while at 2·8 per cent the sugar had no effect. There is, therefore, no disagreement between Linford and Cooke on this point. It is interesting that Feder[40], working on citrus eelworms in Florida, found that sugar was itself an efficient soil nematicide if used in sufficient concentration.

In a second experiment along the same lines, Linford investigated the effect of varying quantities of chopped pineapple leaf added to the soil.

To 2,400-g quantities of soil he added respectively 100, 200, 300 and 400 g of chopped pineapple leaf. Using cowpeas as indicator plants he found that the galling of the roots by root-knot eelworm was progressively less with increasing amounts of organic matter up to 300 g, but that the 400-g dose was less effective in protecting the plants than 300 g. This suggestion that there might be an optimum concentration of organic matter was confirmed by Cooke, who showed that a similar optimum existed when chopped cabbage leaf was used as a soil amendment.

In further experiments Linford compared the effects of finely and coarsely chopped pineapple leaves, and also of fresh and dried leaves. He found little evidence that finely chopped leaves differed in their effect from coarsely chopped, or that fresh leaves differed from dried leaves. In a final experiment he endeavoured to find out whether the reduction in eelworm numbers as a result of adding chopped pineapple leaves to the soil was a sudden effect or whether it took place gradually: this experiment was somewhat less satisfactory than the others, but he obtained indications that the peak of fungal activity was reached early in the process of decomposition of the pineapple leaf, and that it dried away fairly quickly. This finding agrees with the observations of Cooke.

The work of Linford and his colleagues is most interesting, and it has paved the way for further work on the use of predacious fungi for the biological control of eelworms. One word of warning must be given, however. Linford showed that green manuring increased the eelworm population in his experimental soil, that the activity of predacious fungi in the soil was stimulated, and that eventually the eelworm population fell, with the result that eelworm damage to plants was lessened. He did not show, however, that the control of the eelworm was directly attributable to the fungi. This still remains to be proved.

Linford's work on predacious fungi came to an end shortly before the last war, and has not been resumed. The scene then shifted to France, where, during the war years, a number of workers interested themselves in the use of predacious fungi for the biological control of eelworms[2–7,41–54]. A great deal of this work concerned some of the smaller nematodes parasitic in farm animals, but plant pathogenic eelworms also received their share of attention.

The French work was largely aimed at elucidating some practical problems likely to arise in the biological control of eelworms. Three fungi were used in the experiments: *Arthrobotrys oligospora* (sticky networks), *Dactylella ellipsospora* (sticky knobs) and *D. bembicodes* (constricting rings). Laboratory tests were made to make sure that these fungi were able to capture and destroy some of the nematodes that parasitize farm animals, and methods were worked out for culturing the fungi in bulk for soil inoculation, and for harvesting and drying their spores. It was also shown that the fungi were harmless to animals when cultures were fed to them, and that they were without parasitic action on plants, an important point if they are to be used in agriculture for eelworm control.

Two experiments were made in France on the practical control of parasitic eelworms, but both were inconclusive. The first concerned nematodes attacking sheep. Two similar enclosures were set up in a pasture, both of which were artificially infested with *Strongyloides papillosum* and a species of

Bunostomum. One enclosure was then treated with spores of predacious fungi. Two healthy 10-months-old lambs were put into each enclosure and allowed to graze. After 5 weeks the lambs were removed to the Pasteur Institute and kept under observation. The lambs from the fungus-treated enclosure remained healthy, while those from the enclosure that was not treated with fungi were found to be infected with eelworm. This result was interesting, but with only two lambs in each pasture no definite conclusions can be drawn from it. It is a pity that more animals were not used.

The second trial was carried out in Paris against root-knot eelworm in begonias. Two fungi were used: *Arthrobotrys oligospora* and *Dactylella bembicodes*. Thirty-nine pots were set up, of which 10 received spores of *Arthrobotrys oligospora* and 11 were treated with spores of *Dactylella bembicodes*: 18 were left untreated. Begonia cuttings were planted in all the pots and left to grow from March until late September; the plants were then lifted, and the results of the treatments were assessed by counting the number of plants that showed galling of the roots, and also by counting the actual number of galls on each root system. Of the plants given fungal protection, three out of 21 were infected, the total number of eelworm galls being five, while of the control plants eight out of 18 were infected, the total number of galls being 85. The figures for numbers of infected plants do not differ significantly between the treatments: the figures for total numbers of galls are more impressive, but the authors do not quote sufficient data to allow statistical analysis, so the result of the experiment must be regarded as indeterminate.

The French work ended in 1945, and does not seem to have been taken up again. This is unfortunate, for the results obtained were both interesting and valuable.

Work in this country on the biological control of nematodes began in 1951, with experiments on the control of the potato root eelworm (*Heterodera rostochiensis*). Like all species of *Heterodera*, this is a cyst-forming eelworm: the body of the female, after fertilization in the roots of the host plant, becomes a resistant cyst, filled with eggs, that is left in the soil, where it may remain viable for many years. The cyst-forming habit makes these eelworms very difficult to deal with, as the cysts are resistant to attack, and their long period of viability in the soil is an obstacle to the common sanitary practice of starving the eelworm into submission by crop rotation. A survey carried out shortly after the last war estimated the annual loss of potatoes from potato root eelworm in Britain alone at about 250,000 tons, with a cash value in the region of £2,000,000, and, as no economic method of control on a field scale has yet been devised, it is unlikely that the position has improved since then.

The presence of cysts in infested soil provides a ready means of estimating the level of eelworm population, and hence the potential danger to a potato crop. The cysts can be extracted from soil samples by a simple flotation technique[54], and the eelworm population expressed either in terms of the number of viable cysts per gramme of soil, or as the number of larvae obtained from the cysts.

In an initial series of experiments by Duddington and Dixon, potato plants were grown in flower pots in soil infested with potato root eelworm

(*Heterodera restochiensis*), the cyst population of the soil being known. Several different nematode-trapping Hyphomycetes were used, and various methods of applying the fungus to the soil were tried. In some cases an organic soil amendment, such as sterile leaf-mould or compost, was added to the fungal treatments. The results of the experiments were assessed by pre- and post-cropping cyst and larval counts from samples of the soil in the pots. The final results were unequivocal but somewhat mystifying. In all treatments where the fungi had been used without organic amendment there was little or no effect on the final cyst and larval populations, but in every case where organic amendment had been used in addition to predacious fungi there was a startling rise in cyst and larval counts.

These results were difficult to explain, for it seemed hardly credible that the eelworms could actually have benefitted from their exposure to predacious fungi. A later field trial by Brooke, Duddington and Juniper, carried out in Lincolnshire, provided a possible clue. In this trial, potatoes were grown in a field heavily infested with potato root eelworm, and three treatments were given. These were: farmyard manure inoculated with *Dactylaria thaumasia*, farmyard manure from the same source but not inoculated, sand and maizemeal cultures of *D. thaumasia* without the addition of any organic manure; a fourth untreated plot acted as control. Only the rows treated with inoculated dung produced a normal crop. Where uninoculated dung was used the crop was badly damaged by eelworm, with many gaps, and the yield was at the rate of about 4 tons/acre. In the rows treated with sand and maizemeal cultures, and in the untreated control rows, the crop failed completely.

These results were striking, but the post-cropping cyst counts told a different story. The highest cyst count came from the soil that had been treated with inoculated dung: next came the soil that had been given uninoculated dung, followed by the soil receiving sand and maizemeal cultures, the lowest cyst count being that given by the untreated control. There is a notable similarity between these results and those of the pot experiments.

If we make the assumption that the protective effect of the inoculated dung was, in fact, due to the action of the fungus, a possible explanation of the cyst count anomaly suggests itself. It is known that potatoes can give a satisfactory crop in land heavily infested with eelworm if the young plants can become well established before the main eelworm invasion of the roots takes place. Linford has suggested, and Cooke has confirmed, that when predacious fungi are activated in the soil by the presence of organic matter, the initial period of intense activity is short. In the Lincolnshire field trial, as well as in the pot experiments, it is at least possible that the fungi, in conjunction with the added organic matter, gave an initial protection to the potato plants that enabled them to establish themselves, and that the subsequent fall in fungal activity left them open to attack by eelworms. If the initial protection were sufficient to get the plants well established, their extensive root systems would provide a larger surface for the subsequent formation of eelworm cysts than the relatively poor root systems of the plants that had been subject to eelworm attack from the outset. This is but a tentative explanation—some would say an optimistic one—for which there

is no justification except plausibility. It is hoped, if opportunity presents itself, to repeat the pot experiments under conditions where measurements of yield, extent of root system and so on can be made, in order to settle the question.

The result of the initial potato trial in Lincolnshire was encouraging, but further trials failed to produce confirmation. In a microplot experiment at Cambridge[57] the result was indeterminate as the eelworm failed to attack, the plants in both treated and control plots remained healthy; a second potato trial in Lincolnshire on a more extended scale met with the same fate. Two further potato trials were carried out in Lincolnshire: one of these failed as the crop was destroyed by drought, while the result of the other was indeterminate.

The sugar beet eelworm (*Heterodera schachtii*) is a serious pest of sugar beet in some parts of the country. Little work has been done on the biological control of this species, but a microplot experiment carried out at Cambridge[58] produced increased yields varying from 30 to 50 per cent following the application of predacious fungi combined with organic amendment of the soil, although no reduction in the cyst and larval populations of the soil was observed.

During the past few years work has been in progress on the biological control of the cereal root eelworm (*Heterodera avenae*). This eelworm has received little publicity, since in this country it has mainly attacked oats, which are not a cash crop, but its incidence is increasing and it has become a serious worry to many farmers, especially in the west. In recent years, too, it has shown an increasing tendency to attack oats. Work on the biological control of this eelworm began in 1954 at the Wolverhampton headquarters of the National Agricultural Advisory Service, West Midland Region, and has been continued at the Southern Region headquarters at Reading.

The first experiments in this series were designed to confirm Linford's observations on the effects of green manuring. Oats were grown in microplots in which the soil had been artificially infested with eelworm, the only treatment given being green manuring with chopped cabbage leaves[38]. The effect of the treatment was assessed by lifting oat seedlings about 3 weeks after sowing and counting the numbers of eelworms in the root cortex, after staining with acid fuchsin in lactophenol. At the time of lifting, soil samples were examined for predacious fungi, and the activity of the fungi was estimated according to an arbitrary scale. The mean number of *Heterodera avenae* per root system in the treated plots was 12·3, compared with 31·8 for the controls. There was, however, considerable variation between plots, and the mean difference, 19·5 $\pm$ 9·98, just failed to reach significance at $p = 0{\cdot}02$. There were indications of greater fungal activity in the treated plots, but the techniques then available for the estimation of the activity of predacious fungi in soil were too crude to give a reliable quantitative result. A repetition of the experiment, using the same methods, gave a mean number of eelworm larvae per root system of 14·4 for the treated plots as against 23·6 for the controls. The mean difference of 9·2 $\pm$ 3·0 was significant with a probability of less than 0·02.

The oats experiments were next extended to include inoculation of the soil with a predacious fungus as well as green manuring[39]. Three treatments

were given: mycelium of *Dactylaria thaumasia* with grass mowings, mycelium of *D. thaumasia* alone, grass mowings alone; a fourth untreated plot acted as control. The effects of the treatments were assessed as before by counting the eelworm larvae in the roots of sample seedlings, the counts being expressed as eelworms per gramme of root. In the first trial in this series the mean numbers of eelworm larvae per gramme of root from the four plots were as follows:

Fungus and grass mowings	98
Fungus only	97
Grass mowings only	157
Untreated	235
Significant difference ($p = 0{\cdot}05$)	55·5

It will be seen that where fungus was used, with or without the addition of grass mowings, the eelworm invasion of the roots was significantly reduced compared with the plots where grass mowings were used alone, and that all three treatments gave significantly lower invasion figures than the untreated control.

In the light of the previous experiments the failure to distinguish between the effects of the fungus with and without the addition of green manure was a little surprising, especially as the invasion counts for grass mowings alone differed significantly from the control counts. The experiment was therefore repeated, this time in a field at Pattingham, Salop, already infested with cereal root eelworm, using chopped cabbage leaves as the organic amendment. In this trial, the control plots were given autoclaved fungus mycelium to ensure that the figures were not biased by the fungus itself acting as a form of organic manure. The figures for the mean number of eelworms per gramme of root were as follows:

Live mycelium with chopped cabbage	127
Live mycelium only	254
Autoclaved mycelium with chopped cabbage	370
Autoclaved mycelium only	408
Significant difference ($p = 0{\cdot}001$)	110·5

Here the first three treatments differ significantly from one another, and the first two differ from the control with a probability of 0·001.

These experiments are being continued to ascertain the relative effects of predacious fungi with different types of trapping mechanism, and of different species with similar traps, on the invasion of oat roots by cereal root eelworm. Recent work by Duthoit and Godfrey, using *Arthrobotrys oligospora*, *A. robusta* and *A. conoides*, all fungi with sticky networks, indicates that there may be considerable differences in the efficacy of different species, even though their traps may be morphologically similar.

Some interesting work has recently been published by Hams and Wilkin[59]. They successfully grew a number of predacious fungi, including *Arthrobotrys robusta*, *Dactylaria candida* and *Trichothecium cystosporium*, in submerged culture, using a liquid medium containing whey powder, lactose, corn steep liquor, potassium phosphate and water, with the pH adjusted to 6·8 to 7·0. Growth and sporulation were good, and by the use of vermiculite they were able to obtain their fungi in the form of air- or vacuum-dried preparations that

could be stored until they were needed for soil inoculation. The dried preparations were assayed for activity by pot tests on oat seedlings grown in soil infested with cereal root eelworm, the potency of the fungus preparations being judged by counting the eelworm larvae that had invaded the roots of the sample seedlings. Besides using nematode-trapping fungi, Hams and Wilkin experimented with two fungi, *Cylindrocarpon radicicola* and *Phialophora heteroderae*, that attack eelworm cysts in the soil.

In order to study the influence of various factors on the activity of the fungi in soil, Hams and Wilkin used an ingenious method based on respirometric measurements. Sterilized soil was inoculated with the fungi and placed, with phosphate buffer, in a Warburg respirometer. The level of respiration was then observed under varying conditions of pH and moisture content, as well as in the presence of varying doses of agricultural fertilizers. The persistence of growth in the soil was also measured in terms of respiratory activity.

Using *Cylindrocarpon radicicola* as a test fungus, Hams and Wilkin found that variation of the soil pH between 5·0 and 8·0 had little effect on oxygen uptake. Respiratory activity increased with increasing amounts of water in the soil up to a point, but fell away again as the soil approached saturation. The addition of agricultural fertilizers to the soil had no beneficial effect on the fungus.

In addition to this fundamental work, Hams and Wilkin carried out an extensive series of pot and field experiments on eelworm control by predacious fungi. In 1956 a large pot trial on potato root eelworm in potatoes and tomatoes, and pea root eelworm in peas, was set up, with *Dactylaria thaumasia*, *Trichothecium cystosporium* and *Arthrobotrys robusta* as experimental fungi. The effect on peas was particularly striking, the yield being increased ninefold by the use of *A. robusta*. The authors do not quote the figures for potatoes and tomatoes, but they state that the effect of the fungi on these was less well defined.

The following year, field trials were laid out in which 'resistant' spore forms of *Arthrobotrys robusta* and *Dactylaria candida* were used, as well as filter-dried mycelium of *A. robusta*. Potatoes and peas were the experimental plants, the respective eelworms being *Heterodera rostochiensis* and the pea root eelworm, *H. gottingiana*. Various organic soil amendments were tried, including bran, farmyard manure, and chopped cabbage leaves. Assessing the results of the trials by crop yields, it was found that a significant increase in yield of potatoes was obtained using *Arthrobotrys robusta* alone or with chopped cabbage, and *Dactylaria candida* with chopped cabbage. All other treatments failed to give significant yield increases, and of the experiments with peas, none showed a significant result.

In 1958 further trials were carried out on cereal root eelworm in oats and potato root eelworm in potatoes. Three fungi, *Dactylaria candida*, *Trichothecium cystosporium* and *Phialophora heteroderae* (a cyst-attacking fungus) were used in the form of 'spore' preparations on vermiculite or kieselguhr, as well as dung inoculated with *Dactylaria candida* and *Trichothecium cystosporium*. The results of these trials were entirely negative, since any increase in crop could have been attributed to the farmyard manure used as organic amendment.

Finally, in 1959 Hams and Wilkin set up a pot trial on cereal root eelworm in oats, using *Cylindrocarpon radicicola* as test fungus. The results were assessed by counting the larval invasion of the roots of the oat seedlings, and were entirely negative. This is hardly surprising, since *C. radicicola*, as far as is known, only attacks eelworm cysts: it could scarcely have been expected to affect the larval emergence during the short time of the experiment. It is a little difficult to understand why a fungus of this type was chosen for a short-term experiment.

These extensive experiments by Hams and Wilkin are both interesting and valuable, even if their results were disappointing in their failure to achieve any significant control of eelworm, except in a few instances. In this the field trials differ notably from the preliminary pot experiments, which were encouraging. The authors point out, with considerable justification, that in pot trials it is possible to adjust the conditions to suit the fungi, whereas in field trials it is not. They also point out the importance of synchronizing the peak of fungal activity with the peak emergence of eelworm larvae from the cysts. In dealing with a species of *Heterodera* it must be remembered that the emerging larvae may have only a few millimeters to travel from the cyst to the sanctuary of the host root, and it is only during this brief journey that they are in any serious danger from the fungi.

One reason for the failure of Hams and Wilkin to obtain any substantial reduction in eelworm attack may have been that they were using dried preparations of fungi. Their plots, too, were rather small for an experiment intended to be judged by yield, and it is a pity that more use was not made of the technique of counting the larvae invading the roots of test plants. In this connexion, it is interesting that the second oats trial by Duddington, Duthoit and Everard[39], which gave a very highly significant result using living fungus mycelium, was carried out in the same field, and at the same time, as one of the negative 1958 oats trials of Hams and Wilkins.

In discussing their results, Hams and Wilkin suggest two possible conclusions: '(*a*) that either the experiments described have not solved the problem of the correct use of predacious fungi for the field control of eelworms, or (*b*) that the results obtained suggest that this type of biological control is ineffective on a practical scale'. The truth of the first conclusion is self-evident: the second is a little ambiguous in its wording. If 'this type of biological control' refers to the use of dried preparations of the kind employed by the authors, the conclusion may be justified, though a little pessimistic. If, on the other hand, 'this type of biological control' refers to the use of predacious fungi in general, then the conclusion is not only ahead of the evidence, but is also contrary to the findings of other workers.

During the last decade a great deal of work has been done on predacious fungi in the U.S.S.R., and especially in Turkmenistan. Soprunov and Galiulina[60,61] have described a number of new species of nematode-trapping Hyphomycetes, and some interesting and important work has been done on the biological control of nematodes. In a recent paper[62] Soprunov and Tendetnik state that, as a result of 5 years of testing, they have concluded that the two most promising fungi for eelworm control are *Arthrobotrys oligospora* and *A. dolioformis*. They consider that the best culture substrates are chopped maize and oatmeal, cultures on which can be dried and ground,

a powder containing up to 2,000,000 spores per gramme costing about 4·50 roubles a kilogramme. The powder can be applied to the surface of the soil or mixed with 1,000 times its weight of soil or manure. They claim that a fungus applied in this way remains viable in the soil for a year, though a marked falling-off occurs in from 3 to 4 months after its application.

Other work has been carried out in the U.S.S.R., but details are hard to come by. This interesting work has, however, recently been reviewed in a book by Soprunov[63].

During the last few years the possibility of using predacious fungi for the control of eelworms has aroused much interest in the U.S.A., and active experimentation is now going on, with which the names of Feder and of Tarjan are particularly associated. Full details are not yet to hand, but it is likely that interesting and important communications will come from across the Atlantic before very long. Compared with the U.S.A. and U.S.S.R., this country is lagging behind badly in the field of biological control of eelworms.

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THE PHYSIOLOGY OF ABSORPTION FROM THE ALIMENTARY CANAL IN INSECTS

J. E. TREHERNE

INTRODUCTION

THE absorption of nutrient substances from the alimentary canal has been a classical subject for research in mammalian physiology. The equivalent processes in other animal groups, however, have been little studied. Thus, although a fairly extensive body of information has accumulated dealing with digestion and nutrition in invertebrates, the related processes of absorption have, in general, remained largely unknown. Recently, however, with the advent of new physical and biochemical techniques some investigations have been carried out on the physiology of the various absorptive processes in insects. This research on species from the largest of the animal groups, taken with some earlier qualitative work, has resulted in the emergence of a body of information sufficient to enable this topic to be adequately reviewed for the first time.

The absorption of organic materials from the alimentary canal in insects represents the culmination of a whole series of physiological events. It is, therefore, unprofitable to consider the absorption of organic molecules by the gut epithelium without relation to the preceding digestive processes. In this review the uptake of these compounds is thus related to digestion by a short introductory account of these processes for each class of the organic materials.

THE STRUCTURE OF THE INSECT GUT

As would be expected in such an enormous group, which includes species capable of feeding on almost every kind of organic material, the alimentary canal in insects shows a striking degree of structural variation and complexity. The generalized anatomy of the insect alimentary canal is usually explained by reference to the relatively unspecialized structures in such species as cockroaches and certain Orthoptera (*Figure 5.1*). In these forms the foregut is enlarged to form a muscular crop, with associated proventriculus, the lumen of which is covered with a cuticle continuous with that of the general body surface. The proventriculus appears to have a function similar to that of the pylorus in mammals in controlling the rate of passage of food into the midgut and restricting its regurgitation into the crop.

The midgut region of the alimentary canal consists of the tubular ventriculus and a variable number of diverticula or caeca which open into the lumen at its anterior end. The epithelium of the midgut, which is of endodermal origin, is composed of cubical or columnar cells with an

associated basement membrane, circular and longitudinal muscles (*Figure 5.2*). The midgut epithelium does not possess a cuticular lining but is overlain by the chitinous peritrophic membrane which is secreted by groups of cells at the anterior limit of the midgut or, as in many insects, by delamination from the general midgut epithelium. It is generally believed that the peritrophic membrane functions to protect the cells of the midgut from abrasion and damage, thus replacing the protective mucous secreted in the mammalian gut[2]. The permeability properties of this membrane appear to have little significance as far as the absorption of physiologically

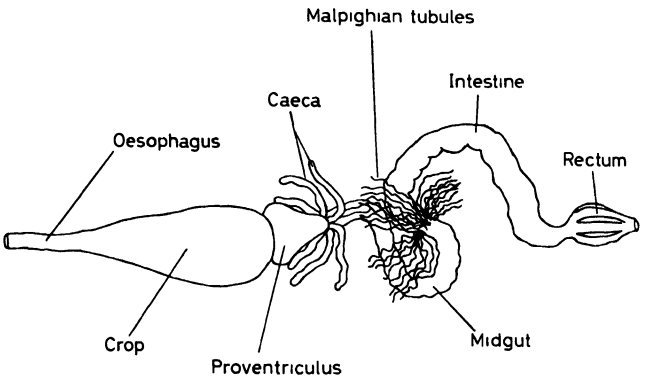


Figure 5.1. The alimentary canal of the cockroach *Periplaneta americana*

important molecules are concerned, for only large colloidal particles are restrained by it. In the larva of the mosquito, for example, colloidal gold particles of 2 to 4 μ diffuse through the peritrophic membrane although those of 20 μ diameter are excluded[3].

In the hind gut, which receives material discharged from the midgut and Malpighian tubules, the epithelium is of ectodermal origin and is consequently lined with a layer of cuticle. Unlike that lining the foregut this cuticle is readily permeable to water[4,5]. The terminal portion of the hind gut is enlarged to form a rectum. The cells in this region of the hind gut tend to increase in size and to form a number of internal thickenings, the rectal pads or papillae (*Figure 5.2*).

In some primitive insects, such as Collembolans, the alimentary canal differs from that outlined above in showing very little differentiation apart from the possession of proctodeum, mesenteron and stomodeum. Other more advanced groups, on the other hand, show many deviations from the generalized structure of the alimentary canal. In the foregut, for example, many Diptera and Lepidoptera have crops formed as lateral diverticula of the oesophagus. In mosquitoes such diverticula are specialized for the storage of sugary fluids, the blood meals being passed on directly to the midgut[6]. In some other blood-sucking forms such as *Cimex* and *Rhodnius* the midgut has become modified to serve the function of a crop. Other modifications can be found in the Homoptera where the posterior portion

of the midgut is intimately related to the terminal part of the oesophagus or the first portion of the midgut, while in Coccids some coils of the midgut are completely enveloped by the hind gut. Such structures are generally believed to be mechanisms enabling excess fluid in the diet to by-pass some portion of the alimentary canal[2].

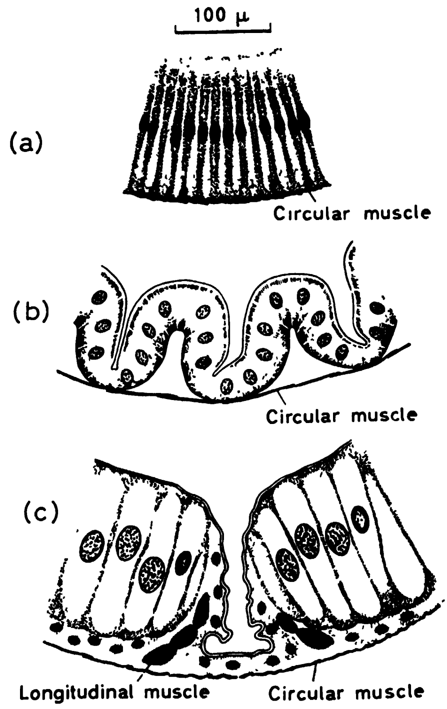


Figure 5.2. The structure of the gut wall from different regions of the alimentary canal of the stick insect *Dixippus morosus*[1]

More exhaustive discussions of the structural variations of the alimentary canal in insects are given by Snodgrass[7] and Wigglesworth[2].

ABSORPTION OF SUGARS

Carbohydrate Digestion

Most of the insects studied have been found to possess the enzymes necessary for the reduction of carbohydrates to their component sugars. The hydrolysis of the complex polysaccharide molecules of starch and glycogen is effected in many insects by amylase. In the cockroach, for example, this enzyme is exceptionally active in the saliva[8] and is also abundant in the midgut secretions and saliva of such species as the blow-fly *Calliphora*[9], the silkworm

Bombyx mori[10] and various species of beetle[11,12]. Amylase is absent, or present in only very small quantities, in such insects as the larvae of the blow-fly *Lucilia*[13] and the Lepidopteran *Lespeyresia*[14] which feed on rather limited diets. As in other organisms two distinct fractions are thought to occur in insect amylase. The α-amylase, or 'dextrogenic', component of the enzyme breaks down glycosidic linkages in the interior of the polysaccharide chain with a release of oligosaccharides which are reduced to maltose and glucose relatively slowly. The β-amylase, or 'saccharogenic', component effects a rapid production of maltose by the hydrolysis of the straight chains of glucose units. In the cockroach it seems that the two forms of amylase are present in approximately the same proportion as in human saliva[8]. As with vertebrates the insect amylases are inactivated by dialysis and reactivated by the presence of chloride ions[8,15].

Unlike the higher animals some insects are able to utilize the plant polysaccharide cellulose. The termites, for example, are well known for their ability to digest cellulose[16], depending upon the bacteria and protozoa of the gut for the production of the necessary cellulase. On the other hand, a limited number of beetle larvae from the families Cerambycidae and Anobiidae have been shown to secrete a cellulose digesting enzyme in the midgut[17,18]. The silverfish, *Ctenolepisma lineata*, has also been found able to hydrolyse completely ^{14}C-labelled cellulose, an ability which cannot be attributed to the activities of the gut bacteria[19].

The maltase and dextrin molecules produced by the action of amylase, together with ingested simple glycosides and oligosaccharides, are subjected to further hydrolysis by the various insect glycosidases. The α-glycosidic linkages of the disaccharide molecules of maltose, trehalose and sucrose and of such trisaccharides as melezitose and raffinose are frequently hydrolysed by the gut enzymes of insects (*cf.* Gilmour[20]). The insect invertases, which catalyse the degradation of sucrose to glucose and fructose, seem to be similar to that of vertebrates in being α-glucosidases, although evidence has been obtained that in the midgut of the blow-fly *Calliphora* a weak β-fructosidase may also be involved in the hydrolysis[21]. Among the β-glucosidases secreted by insects is an enzyme which has been shown to be capable of hydrolysing the disaccharide cellobiose produced from cellulose in the gut of *Ctenolepisma*, thus completing the degradation of the polysaccharide[18]. α-Galactosidases have been shown to be present in some insects being able, for example, to effect hydrolysis of melibiose in both larvae and adults of *Calliphora*[21,22]. The β-galactosidase lactase although absent in some species is able to release the constituent galactose and glucose components of the disaccharide molecule in several insects including the silkworm *Bombyx mori*[23], the cockroach *Periplaneta americana*[8] and the larvae of some woodboring beetles[11].

The combination of carbohydrase enzymes, briefly outlined above, associated with any particular species ensures that in most cases a significant proportion of the ingested carbohydrates are reduced to their constituent sugars, most frequently hexose monosaccharides, and are thus made available for absorption from the lumen of the alimentary canal. The various processes involved in the absorption of these substances will be considered in some detail in the succeeding section.

Absorptive Processes

The absorption of the products of carbohydrate digestion has been studied in detail in relatively few insect species. A good deal of the early work on this topic was carried out on various species of cockroaches. One of the features of these investigations was the controversy as to the role of the crop in the absorption of sugars by these insects. Both Plateau[24] and de Bellesme[25] considered the crop to be the principal organ for the absorption of sugars and some other nutrient substances. Cuénot[26] and Biedermann[27] sought to disprove this hypothesis largely on the *a priori* assumption that the chitinous intima of the cells of the crop wall was an effective diffusion barrier to water-soluble substances. The hypothesis was, however, accepted by Jordan[28] and the subsequent researches of Sanford[29] also lent support to the view that the crop was of importance in sugar absorption. The qualitative experiments of Sanford showed that sugars could not be detected in the midgut region of *Periplaneta americana* and it was concluded that these substances did not reach this part of the alimentary canal in significant amounts. The sugars in the crop were, however, clearly demonstrated to decrease and it was again argued that they were, in fact, being absorbed by this organ. The controversy continued a few years later when Abbott[4], working on the Australian roach (*P. australasiae*), found that the crop wall in isolated preparations was relatively impermeable to glucose molecules. These experiments on the isolated crops were, however, carried out on preparations maintained in distilled water and were therefore open to the objection that they were not in a normal physiological condition. Thus, nearly 80 years after the original work of Plateau, some reviewers were not able to resolve this controversy over the absorptive function of the crop[30].

More recently the problem of the absorptive function of the cockroach crop was reinvestigated using ^{14}C-labelled glucose[31]. In these experiments the radioactive glucose was fed to the insects together with a dye, Amaranth, which was not absorbed from the lumen of the alimentary canal or adsorbed in significant amounts on to the gut wall. The net absorption of the labelled glucose molecules was then calculated from the glucose-dye ratio in the various parts of the gut. *Figure 5.3* illustrates the amount of glucose absorbed as the experimental solution reached the different regions of the gut after varying time intervals. It is clear that most of the glucose had disappeared by the time the solution had reached the caeca and the anterior part of the midgut. There was no measurable uptake of radioactive glucose molecules from the lumen of the crop in any of the insects used in these experiments.

In such a system outlined above it is of importance to know something of the rate at which the glucose solution reached the absorptive surfaces in the midgut region of the alimentary canal. Some light was thrown on this process by studying the rate of crop emptying in this insect. *Figure 5.4* is a plot of the volume of the crop contents at varying time intervals after feeding starved individuals with solutions of three different glucose concentrations. The rate of crop emptying, which was an exponential function of time, was considerably slower at the higher glucose concentrations. The rate of crop emptying was, in fact, shown to be linearly related to the concentration of the ingested glucose solution, so that the amount of fluid

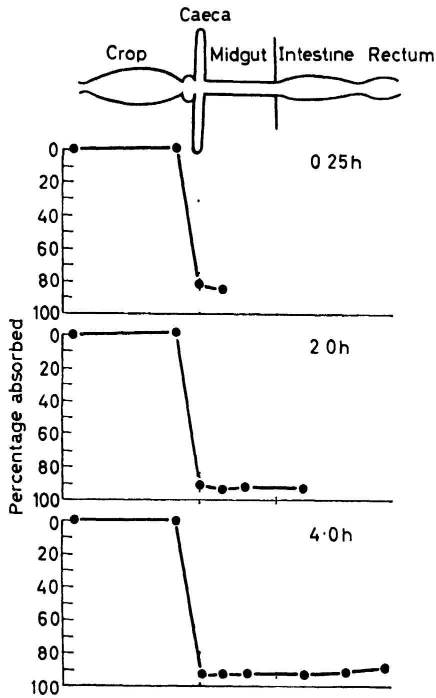


Figure 5.3. Percentage absorption of ^{14}C-labelled glucose from the alimentary canal of the cockroach *P. americana* at varying time intervals after the ingestion of the experimental solution[31]

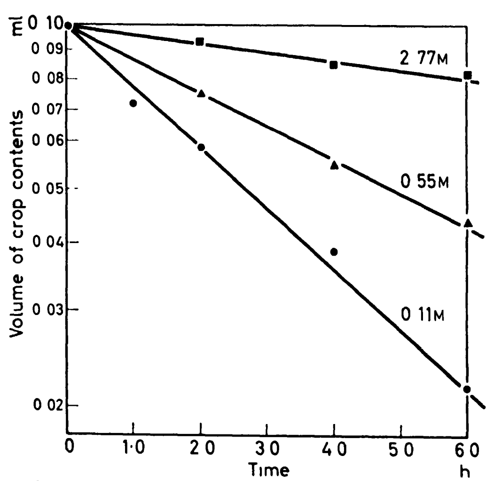


Figure 5.4. Effect of glucose concentration on the rate of crop emptying in the cockroach[31]

leaving the crop decreased with increasing concentration[31]. Thus, the limiting process in the absorption of glucose was not the transfer across the gut wall but the rate at which the fluid was allowed to leave the crop. Such a system would presumably operate to prevent the saturation of any glucose absorbing mechanism in the midgut. It will now be appreciated why some of the earlier workers considered that the crop was the principal organ for sugar absorption in cockroaches. Their qualitative methods showed that the sugar could not be detected in the midgut although that in the crop clearly decreased. The more recent work has shown that the absorption in the midgut is so rapid that the concentration in this region will always be very low, while there is a continuous decrease in the crop sugars as the substances pass into the midgut. The possibility that this might be the explanation was appreciated by Cuénot[26]. A recent investigation demonstrating a conversion of fructose to glucose in the lumen of the gut of the cockroach has also shown that the monosaccharides are absorbed largely in the caeca and the anterior part of the midgut[32]. In addition, ^{14}C-labelled glucose, mannose and fructose have been found to disappear rapidly in the region of the caeca and midgut of the locust *Schistocerca gregaria*[33,34].

The effect of glucose concentration on the rate of crop emptying in *Periplaneta* would seem to be very largely an osmotic effect for it was shown that the rate of emptying remained constant when widely differing substances were tested at the same osmotic pressure[31]. One effect of this type of control of crop function is that a substance in solution can affect the rate of absorption of a second substance by reducing the volume of fluid available for absorption in the midgut. It was shown, for example, that the addition of 0·2M/l. sodium chloride to the ingested glucose solution resulted in a reduction from an absorption rate of $374{\cdot}8 \pm 21{\cdot}8\,\mu g$ ^{14}C-labelled glucose/hour to one of $149{\cdot}1 \pm 23{\cdot}3\,\mu g$ ^{14}C-labelled glucose/hour. This effect has some important implications in insect physiology. It has been pointed out, for example, that the effect of a particular nutrient in high concentration in limiting the rate of movement of food through the insect gut may give rise to nutrient deficiencies in other components present in concentrations which would otherwise be adequate in a balanced diet[35]. Similarly, this effect may be of some importance in insect toxicology for a component of the diet in high concentration may reduce or delay the toxic effects of ingested poison molecules.

The effect of glucose concentration on the rate of crop emptying in the cockroach would appear to be essentially similar to the state of affairs in mammals. For example, in the rat it has been shown that the rate of gastric emptying decreases with increased glucose concentration[36]. The percentage glucose absorption in the rat was also found to be related to gastric emptying[37]. The cockroach mechanism apparently differs from the mammalian counterpart in that the rate of gastric emptying in the rat cannot be directly related to the osmotic pressure of the ingested solution[36].

The physiological processes involved in the transfer of some hexose monosaccharides across the gut wall have been studied in the adults of the desert locust, *Schistocerca gregaria*[33,34]. In these investigations, as with the earlier experiments with *Periplaneta*, the radioactive sugars were introduced into the gut of this insect, together with the dye Amaranth. The percentage

absorption of the ^{14}C-labelled sugars was determined from the sugar/dye ratio in the various parts of the gut. In experiments in which the sugars were fed by mouth it was demonstrated that there was no significant absorption in the crop, the absorption taking place largely in the caeca and

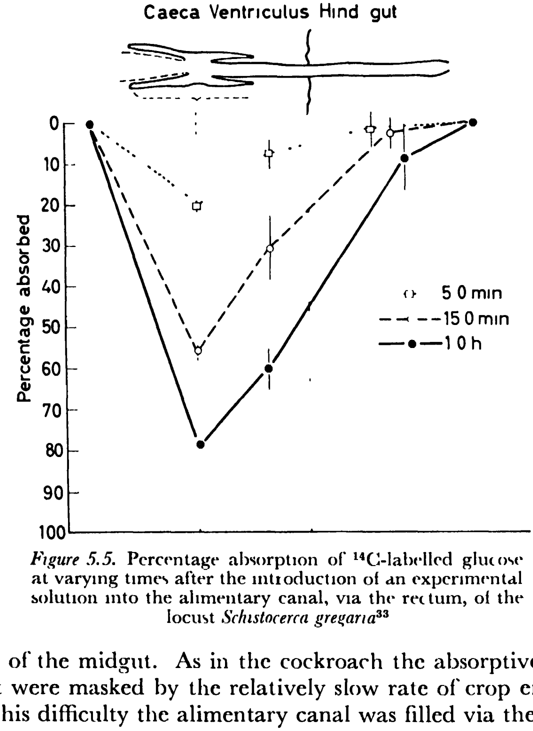
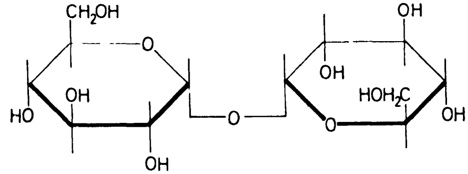


Figure 5.5. Percentage absorption of ^{14}C-labelled glucose at varying times after the introduction of an experimental solution into the alimentary canal, via the rectum, of the locust *Schistocerca gregaria*[33]

ventriculus of the midgut. As in the cockroach the absorptive processes in the midgut were masked by the relatively slow rate of crop emptying. To overcome this difficulty the alimentary canal was filled via the rectum with the radioactive solution, the subsequent absorption of the labelled sugars



(I) Trehalose

being determined. *Figure 5.5* shows the rate of disappearance of ^{14}C-labelled glucose from the alimentary canals of locusts treated in this way. The percentage absorption from the caeca was found to be the same at glucose concentrations of 2·0 and 20·0 mmoles/l., but was reduced at 200·0 mmoles/l. Chromatograms showed that the absorbed glucose had been incorporated as trehalose in the haemolymph. This non-reducing disaccharide (*I*) was

first reported in some species of insects by Wyatt and Kalf[38] and has since been reported in the haemolymph of *Schistocerca gregaria*[39], the blow-fly *Phormia regina*[40] and in *Periplaneta americana*[41]. The experiments showed that at the initial concentration in the gut lumen of 20.0 mmoles/l. virtually all of the absorbed glucose was incorporated as trehalose, but that at 200·0 mmoles/l. relatively large amounts of the monosaccharide accumulated in the haemolymph. These results were not inconsistent with the hypothesis that the limiting factor in the absorption of glucose was a diffusion process. According to this idea it could be expected that the amount of glucose leaving the lumen would be proportional to the concentration difference across the gut wall and for the net percentage absorption to be constant, as indeed it was at 2·0 and 20·0 mmoles/l. At 200·0 mmoles/l. the presence of appreciable amounts of glucose in the haemolymph would tend to reduce the concentration gradient so that the net percentage absorption would be reduced. The conversion of glucose to the disaccharide would, therefore, operate to maintain a steep concentration gradient across the gut wall and thus to facilitate diffusion into the haemolymph. The formation of trehalose involves a virtual doubling of the molecular volume which may tend to restrict back-diffusion into the gut lumen.

A comparative study of the rates of absorption of glucose, fructose and mannose by the caeca of the locust tended to support the hypothesis of absorption by facilitated diffusion[34]. It was found that the rates of conversion of the monosaccharides to trehalose, as measured by the accumulation of the disaccharide in the haemolymph, were very different and paralleled the rates of absorption of the three sugars. Glucose was, at concentrations up to 20·0 mmoles/l. in the gut lumen, converted most rapidly to trehalose and was also absorbed most rapidly. Mannose, which showed a rapid conversion to trehalose only at low concentrations, was absorbed rapidly only at relatively low concentrations. Fructose, which was converted least rapidly of the three sugars, was absorbed at the slowest rate from the gut lumen. The relatively slow absorptions of fructose and mannose from the gut lumen in these experiments can be attributed to the reduced concentration gradient across the gut wall due to the demonstrated accumulation of unconverted monosaccharides in the haemolymph. The sugars were also demonstrated to pass through the gut wall at similar rates in isolated alimentary canals suspended in large volumes of circulating poisoned saline. The passage through the gut wall under these conditions was equivalent to the rapid absorption obtained *in vivo* when there was a rapid conversion to trehalose and again tended to support the hypothesis of absorption by facilitated diffusion.

Analysis of the haemolymph showed that a small amount of glucose (24·1 mg/100 ml.) was in equilibrium with the massive amounts of trehalose in the haemolymph[34,39]. Thus, glucose at very low concentrations in the gut lumen would be at a similar level to the small amount in equilibrium with the trehalose in the haemolymph. Under these conditions a conversion to trehalose could not produce a net movement of glucose molecules into the haemolymph. It was, in fact, demonstrated that at very low concentrations in the gut lumen most of the movement of glucose across the gut wall occurred merely as an exchange with that in the haemolymph[34,42]. With

increasing glucose concentration in the gut lumen the proportion exchanging with that in the haemolymph became progressively smaller, so that the movement of the labelled molecules approximated to the net glucose absorption. The movements of the glucose molecules between the gut lumen and the haemolymph under these conditions are represented diagrammatically in *Figure 5.6.*

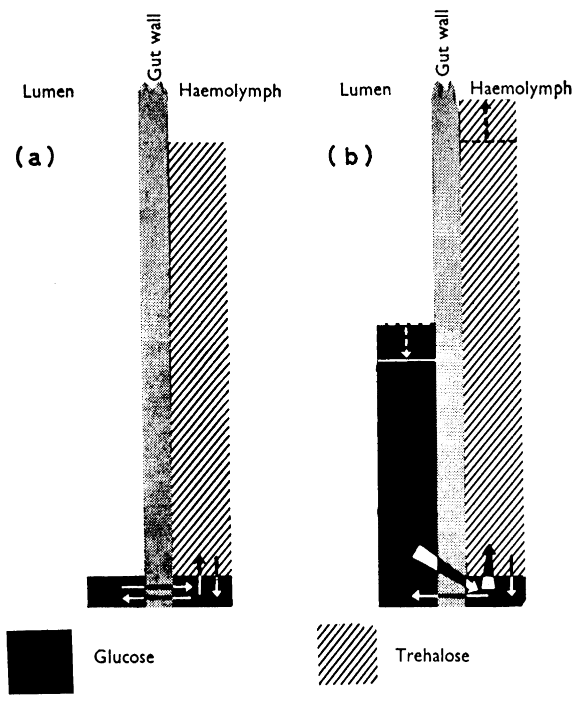


Figure 5.6. Diagrammatic representation of the movements of labelled glucose molecules between the lumen of the caeca and the haemolymph of the locust: (*a*) at low concentration, when the glucose level in the lumen is similar to that in the haemolymph, and (*b*) at relatively high concentration when the glucose in the lumen exceeds that in the haemolymph[34]

The observation by Pillai and Saxena[32] that fructose undergoes conversion to glucose in the alimentary canal of the cockroach is of some importance in relation to the theory of the absorption of monosaccharides, outlined above. It has been mentioned that in the locust fructose is absorbed relatively slowly from the lumen of the alimentary canal. If, therefore, a similar mechanism is responsible for uptake in the cockroach then it would be clearly advantageous to convert a slowly absorbed monosaccharide to glucose in order to hasten absorption in the caeca and midgut. Further, although fructose is present, with some converted glucose, in the gizzard and midgut of the cockroach, the caeca which have been shown to be the principal site of sugar absorption[31], contain only glucose molecules.

The mechanism of monosaccharide absorption by facilitated diffusion is reminiscent of some earlier theories of sugar absorption in mammals. Höber[43] proposed that hexose absorption in the mammalian intestine may be achieved simply by a conversion to some other compound, thus creating a steep diffusion gradient into the mucosal cells. This hypothesis was later developed by Verzár[44] who suggested a possible conversion of glucose to glycogen or a phosphorylation of glucose in the mucosal cell[45]. However, more recent work on mammals has not supported this theory[46,47]. In the locust it has been shown that the conversion to trehalose of ingested glucose does not take place in the cells of the midgut or hind gut of this insect[48]. Incubation of ^{14}C-labelled glucose with fat-body tissue from fifth-instar *Schistocerca* resulted, however, in an appreciable synthesis of the disaccharide. Thus the concentration gradient across the gut wall created by the conversion of glucose to trehalose does not appear to depend upon the metabolism of the tissues of the caeca or midgut. It is perhaps significant in this respect that extensive deposits of fat-body tissue are usually associated with the midgut region of the alimentary canal of *Schistocerca* which may ensure that the absorbed monosaccharide molecules are rapidly converted to trehalose, thus maintaining the necessary low concentration of the monosaccharide in the haemolymph.

The condition of high trehalose and low glucose found in the haemolymph of several species is not a universal occurrence in insects. The haemolymph of the third-instar of the dipterous parasite *Agria affinis* has, for example, been found to maintain about 80 per cent of its carbohydrate as glucose[49]. The larva of the blow-fly *Phormia regina*, in contrast to the adult, also possesses a haemolymph containing a high concentration of glucose[40]. In these species, therefore, some additional mechanism must be postulated to explain the absorption of the dietary monosaccharides.

In the aphid *Tuberolachnus salignus* the greater part of the ingested carbohydrates are excreted[50]. In this and some other species of aphids a proportion of the various dietary sugars are converted to the trisaccharide melezitose (*cf.* Lipke and Fraenkel[51]). In this case it has been suggested that the trisaccharide in the lumen of the aphid gut may possibly play the opposite role to that of trehalose in the haemolymph of *Schistocerca* by restricting the absorption of the unwanted carbohydrates[52].

Some indirect evidence on the absorption of carbohydrates in insects has been obtained from various studies on the histology and histochemistry of the alimentary canal. Wigglesworth[53], for example, studied the cellular pattern of deposition of glycogen reserves in the gut of larvae of the mosquito *Aedes aegypti*. A few hours after feeding starved individuals on starch, heavy deposits of glycogen were found in some cells in the caeca and in the pyloric half of the midgut (*Figure 5.7*). Similar deposits were obtained on feeding glucose, fructose, galactose and trehalose. Only small traces of glycogen were found in the cells of the gut after feeding the pentose xylose and no deposits could be demonstrated after feeding sorbose, arabinose and rhamnose. In the larvae of the blow-fly *Lucilia cuprina* glycogen deposits, localized as uniformly distributed granules, have also been found in the cells of the anterior and posterior zones of the midgut[54]. Such deposits, derived from absorbed substances, are often regarded as the principal sites

of absorption of these compounds in the insect gut (*cf.* Waterhouse and Day[30]). On the other hand, the possibility exists that such deposits are formed, in part at least, from compounds already present in the haemolymph, perhaps absorbed from some other region of the alimentary canal. In the case of *Aedes*, however, the glycogen appeared in the midgut epithelium before the polysaccharide was synthesized in the fat-body and other tissues, and this has been interpreted as evidence of an appreciable direct absorption from the midgut lumen into these cells[53]. In this insect, then, it is possible that the monosaccharides might have been absorbed into the midgut cells

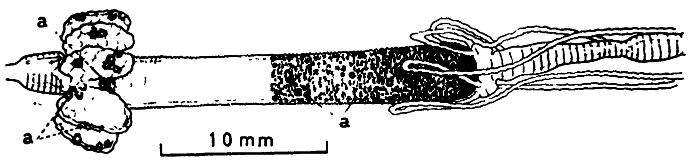


Figure 5.7. The distribution of glycogen (*a*) in the gut of larvae of *Aedes aegypti* after feeding starved individuals with starch or various sugars[53]

due to the steep concentration gradient created by the rapid conversion to the polysaccharide, as was originally suggested by Höber[43] for mammalian intestinal mucosa. It may thus be necessary in absorption studies to distinguish between the proportion of monosaccharides which are translocated and those which are retained by the midgut epithelial cells for metabolism or storage.

ABSORPTION OF FATS

Lipid Digestion

Enzymes capable of hydrolysing the various ingested triglycerides have been demonstrated in the guts of several insect species. As early as 1876 Plateau[24] succeeded in establishing the presence of an active lipase in extracts from the gut of cockroaches. Subsequently Abbott[4], working with *Periplaneta australasiae*, showed that the lipase activity in the crop was due to regurgitation of the enzyme from the midgut region rather than to a secretion by the crop as had been suggested earlier[14,29]. The extracted lipase from *P. americana* was clearly shown to possess an alkaline pH optimum[55]. It has, nevertheless, been more recently shown[56] that *in vivo* the enzyme is apparently capable of appreciable hydrolysis even at the normal crop pH of about 5·0. Triolein digestion was found to proceed more rapidly in well-fed than in starved cockroaches, although in both cases the hydrolysis did not proceed to completion (*Figure 5.8*). Essentially similar results were obtained for the digestion of ^{14}C-labelled tripalmitin by this species, for, 20 hours after feeding, approximately 77 per cent of the triglyceride remained in the unhydrolysed condition[57]. From these investigations it was concluded that the completion of hydrolysis was prevented by the accumulation of the fatty acid within the partly hydrolysed fat. It was suggested that the enzyme was displaced by the end-products of digestion which accumulated at the oil water interface on which the lipase acts and this was confirmed by Frazer's results[58]. It is interesting that the degree of hydrolysis for tripalmitin

was of the same order as that obtained by Frazer with pancreatic lipase when hydrolysis stopped after about 30 per cent of the triglyceride had been digested.

The wax moths, *Galleria mellonella* and *Achroia grisella*, are remarkable for their ability to utilize the beeswax which forms a major constituent of their diet. Approximately half of the ingested wax is absent from the excreta of *Galleria*[59,60]. The wax in the gut of feeding larvae was found to be present in the form of a fine emulsion, presumably as a result of the presence of

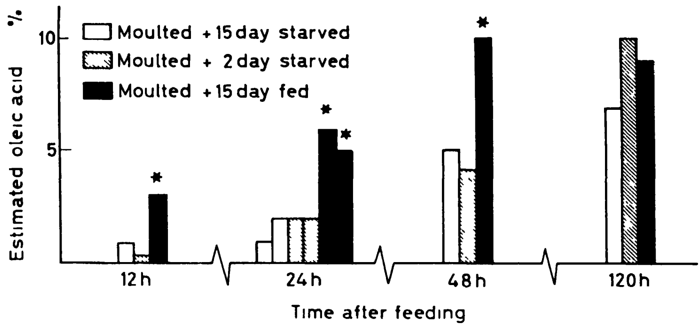


Figure 5.8 The *in vivo* hydrolysis of triolein in the crop of starved and fed cockroaches[56]
* Indicates columns which are significantly different from those in the same group

fatty acids which would be effective in emulsification in the alkaline conditions of the insect's gut. Wax hydrolysis in the alimentary canal was found to occur relatively rapidly, a process which continued in incubated isolated preparations. Analysis of the contents of the gut revealed that, despite the low fatty acid content of beeswax, the lipids from the gut lumen contained as much as 65 per cent fatty acids and only 35 per cent unsaponifiable matter. These authors postulated a conversion of the long-chain alcohols and hydrocarbons to fatty acids in the alimentary canal of the insects. It was also demonstrated that lipids from the lumen had an iodine value of 50, as against that for wax of 15, indicating a desaturation of the wax fatty acids in the lumen of the alimentary canal of *Galleria*[61]. It is not definitely known to what extent micro-organisms are involved in the digestion of wax by *Galleria* and *Achroia*. On the basis of the relatively slow hydrolysis of wax by micro-organisms *in vitro* as compared with the rapid digestion by the insects it has been suggested that these processes may be effected by enzymes secreted by the insects themselves[62].

In many insects in which fats are not a major component of the diet lipase activity may be very weak, as in certain predatory Carabids[63], or virtually absent as in the silkworm, *Bombyx mori*[64].

Absorptive Processes

As with the absorption of sugars the uptake of lipids by the foregut of certain insects has been the subject of some controversy. Plateau[24] concluded from his investigations on the digestive physiology of *Periplaneta americana* that the

crop in this insect was capable of absorbing significant amounts of fatty materials. This view was strengthened by the work of Petrunkevitch[65] in which he demonstrated an accumulation of fat droplets in the crop epithelium following the ingestion of a meal containing fats. Cuénot[26] opposed the hypothesis of the crop as the principal organ of fat absorption, while de Sinéty[66] postulated that the lipid accumulation in the crop epithelium was, in fact, derived from material which had already been absorbed into the haemolymph from some other part of the alimentary canal. These views

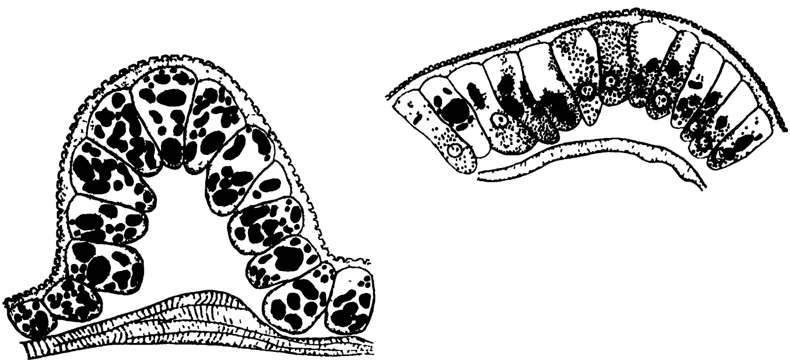


Figure 5.9. Distribution of lipoidal material in the cells of the crop wall of a cockroach after the ingestion of olive oil[29]

were apparently extinguished by most of the subsequent studies made during the following half century. Schluter[67] and Sanford[29], for example, both demonstrated heavy deposits of lipid in the crop epithelium, following the ingestion of fats by cockroaches, which they concluded had entered directly from the lumen of the foregut (*Figure 5.9*). The experiments of Abbott[4], in which he found an accumulation of fat droplets in the crop epithelium even when this organ was ligatured posteriorly, were generally held to have disposed of the suggestion by de Sinéty that the lipids reached the epithelial cells via the haemolymph. More recently Scharrer[68] showed that, in contrast to starved individuals, a considerable amount of fat inclusions were found in the cells of the foregut of the orthopteran *Leucophaea maderae* when fed on a diet containing olive oil. Once again these results were interpreted as showing the crop to be the organ significant in the absorption of lipids.

The whole question of fat absorption in the foregut of *P. americana* was reinvestigated in a very detailed study by Eisner[56]. As has already been mentioned, this author showed that fats are apparently hydrolysed in the lumen of the crop, but that the hydrolysis does not proceed to completion due to the accumulation of the fatty acids within the partly hydrolysed fat. Essentially similar results were obtained using ^{14}C-labelled tripalmitin in this insect[57]. Eisner[56] found that the entry of ingested oleic acid into the crop epithelium occurred relatively rapidly; mineral oils, on the other hand, penetrated much more slowly. Addition of small quantities of oleic acid to the mineral oils, and to olive oil, greatly facilitated the entry of the lipids

into the cells of the crop epithelium. The addition of midgut extracts or crop fluid to the ingested olive oil also increased the number of droplets in the crop epithelial cells. These results clearly show that the addition of a small quantity of fatty acids can greatly facilitate the entry of apolar oils through the cuticular lining of the foregut. Additional lipase, by increasing the rate of degradation of olive oil, resulted in an increased absorption due to the more rapid accumulation of the fatty acids within the oil. Thus it was postulated that the rate of absorption of fats in the foregut depended on both its degree of hydrolysis and upon its viscosity. This partial hydrolysis is assumed to facilitate the entry into the epithelial cells only of the long-chain triglycerides, since digestion of the short-chain triglycerides does not result in an accumulation of fatty acids within the oil.

A quantitative investigation has also been carried out on the absorption of ^{14}C-labelled tripalmitin in the adult *P. americana*[57]. In these experiments, as with those in sugar absorption, the radioactive triglyceride was fed to starved cockroaches together with the dye, Amaranth, the net percentage absorption of the tripalmitin being calculated from the ^{14}C-dye ratio in the various parts of the alimentary canal. The experimental fluid containing the ^{14}C-labelled tripalmitin (palmitate-1-^{14}C) was suspended in a solution of the inert cellulose ester sodium carboxymethyl cellulose dissolved in oleic acid which was subsequently emulsified. Rather unexpectedly, in both cases, it was found that very little absorption appeared to take place from the crop (*Figure 5.10*). There was, however, a very rapid disappearance of the ^{14}C-labelled material when the fluid entered the midgut region. The results indicated that the absorption in the midgut region occurred largely in the caeca and the anterior part of the ventriculus. It was impossible to separate the parts played by these two regions using this technique as the experimental fluid invariably appeared in them simultaneously. These findings do not support the hypothesis that the crop is an important organ in fat absorption. It could conceivably be argued that absorption of a substance such as tripalmitin differed from that of the olive oil used by many of the previous authors. From the observations of Eisner[56], however, it would certainly be expected that the tripalmitin dissolved in oleic acid would be able to enter the cells of the crop epithelium, for he showed that the absorption of oil mixtures is greatly facilitated by the presence of this fatty acid. The fact that the radioactive triglyceride is not absorbed in significant amounts suggested that the droplets of fat observed in the epithelium represent only a very small proportion of the total ingested fat, the greater part of which is absorbed in the anterior part of the midgut region. Wigglesworth[53] has shown that droplets of oil can be made to appear in the cuticular epithelium of *Rhodnius* when oleic acid or olive oil is placed on the abdominal surface. Thus there is no reason to suppose that the crop wall of the cockroach is any more permeable to fats than the rest of the body surface. There is, as well, some evidence that the fat droplets found within the crop epithelium cannot be readily utilized and also cannot be effectively translocated[69]. Thus, although freshly moulted roaches lack fat inclusions in the crop epithelium, adults invariably contain significant deposits of lipids in these cells. Prolonged starvation, of up to 30 days, does not, however, result in a disappearance of the fat inclusions from the crop epithelium.

The above account does not supply any information on the fate of glycerol released on hydrolysis of the tripalmitin, for this portion of the molecule was not labelled with ^{14}C. It seems unlikely that appreciable amounts of glycerol would be absorbed in the foregut of the cockroach. The crop is lined with cuticular wax which is relatively impermeable to water[5] and, by analogy with other water-soluble compounds[70], is likely to be an effective barrier to the diffusion of glycerol into haemolymph.

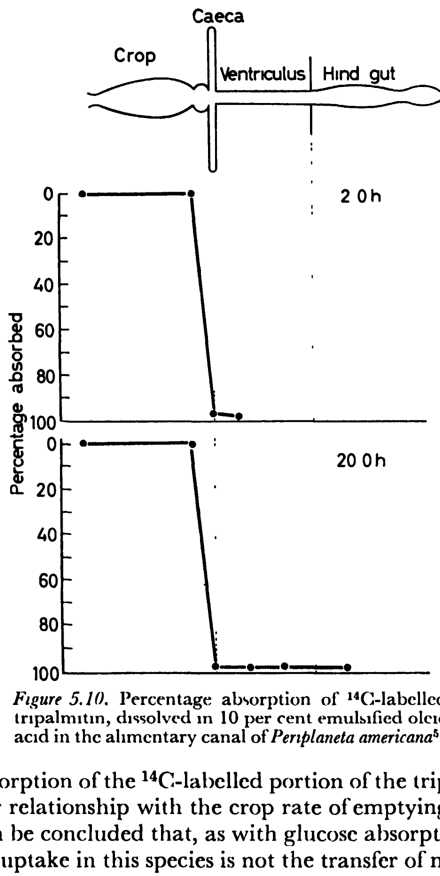


Figure 5.10. Percentage absorption of ^{14}C-labelled tripalmitin, dissolved in 10 per cent emulsified oleic acid in the alimentary canal of *Periplaneta americana*[57]

The total absorption of the ^{14}C-labelled portion of the tripalmitin molecule showed a linear relationship with the crop rate of emptying in *P. americana*[57]. From this it can be concluded that, as with glucose absorption[31], the limiting process in lipid uptake in this species is not the transfer of material across the gut wall, but the rate at which it is allowed to leave the crop. Such a system ensures that lipids will always be at a relatively low concentration in the midgut lumen. This system also effectively masks the process at the site of absorption so that conventional feeding experiments cannot throw a great deal of light on the mechanism of uptake in the midgut.

Our knowledge of the absorptive processes in the midgut is very largely based on various histochemical studies showing the distribution of lipids in the epitheial cells of several insect species. In the mosquito larva, for example, it has been shown that, after feeding starved individuals with olive oil, the

subsequent accumulation of fat droplets was largely confined to scattered cells in the caeca and to the clear cells of the anterior half of the midgut[53]. In the blow-fly, *Lucilia sericata*, it has been claimed that, following ingestion of olive oil, droplets of fat appeared in the cells of the anterior and posterior segments, but were absent from the middle portion of the midgut[13]. A more recent investigation of the distribution of lipids in the alimentary canal of *Lucilia cuprina* has shown that with larvae fed on a balanced diet there were

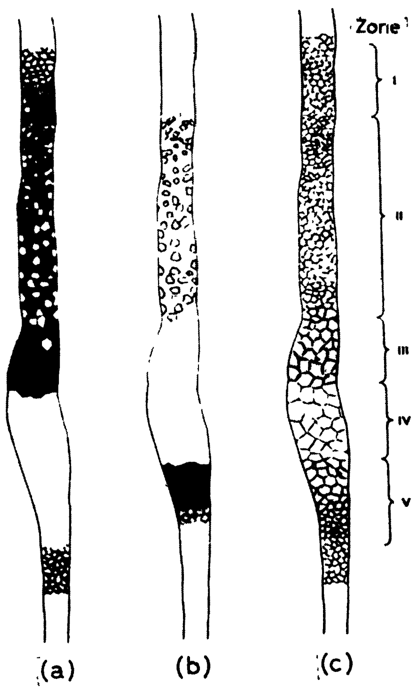


Figure 5.11. Distribution of (*a*) reducing lipoid. (*b*) ferric iron and (*c*) copper in the middle region of the midgut of larvae of the blow-fly *Lucilia cuprina*[54]

extensive deposits of fatty material in the middle region and in the posterior portion of the midgut[54] (*Figure 5.11*). Larvae fed on a liver diet also showed in addition an accumulation of lipids in the anterior portion of the midgut. The cells containing osmic-reducing lipids in the middle portion of the midgut exhibit a most remarkable mosaic pattern which Waterhouse and Stay[54] have divided into five distinct zones (*Figure 5.11*). Electron micrographs of lipophilic cells from the second zone of the middle portion of the midgut show that these structures are unusual in having a striated border composed of lamellae instead of microvilli[71].

The precise role of the lipophilic cells of the midgut in fat absorption has

yet to be established. It is often assumed that such cells are exclusively responsible for any demonstrated absorption of fatty materials from the midgut lumen. This postulate has more basis in fact than that supposing uptake of lipids through the crop wall, for, unlike those in the cockroach crop epithelium, the fat droplets in the cells of the midgut of several insects have been shown to disappear rapidly on starvation and can thus be assumed to have been effectively utilized and to be capable of translocation. There is, however, no evidence as to the proportion of absorbed lipids which can be attributed to a transfer via any particular zone of lipophilic cells in the midgut. It is conceivable, for example, that a significant proportion of emulsified lipids passes through spaces between the epithelial cells, perhaps by some form of interfacial transport of the type postulated by Crisp[72]. Alternatively it is possible that the rate-limiting process in the transfer of lipids across the midgut epithelium to the haemolymph may in some cases be the passage across the cell boundaries adjacent to the gut lumen, so that the actual lipid content of the cell cytoplasm may be relatively low. In both these cases, then, an appreciable transfer of lipid across the midgut epithelium may be effected without any dense accumulation of the substance within the cytoplasm of the cells.

The absorption of the products of wax digestion in the larvae of *Galleria* has been shown to occur relatively rapidly, for, despite the large quantity of food which passes through the gut, only about 50 per cent of the ingested wax is excreted[60]. The work of Wlodawer[61] demonstrated that about 20 per cent of the lipids associated with the alimentary canal were present in the form of phospholipids. Only a small proportion of the phospholipids was found to be present in the gut lumen, the greater part being contained in the walls of the foregut and midgut where it formed as much as 50 per cent of the total lipoidal material. During starvation the phospholipid content of the alimentary canal fell rapidly. Cytochemical investigations also demonstrated the presence of phospholipids in the midgut epithelial cells of feeding larvae, these compounds disappearing in starved individuals[73]. It is concluded from these studies that the products of hydrolytic breakdown of wax formed as a result of the digestive processes, outlined in the preceding section, penetrate the cells of the gut wall in a phosphorylated form. Enzymes capable of dephosphorylating phospholipids have been demonstrated in the gut tissues and it is assumed that the majority of these compounds undergo dephosphorylation in the epithelial cells, the remaining components entering the haemolymph in a relatively unchanged form[61]. These observations seem to parallel those made on mammals in which formation of phospholipids in the intestinal wall has been demonstrated[74], and here, as in *Galleria*, the precise role of the phospholipid formation in fat absorption remains to be discovered.

AMINO ACID AND PROTEIN ABSORPTION

Protein Digestion

It has been pointed out that, in general, the various insect proteinases studied have been found to exhibit many similarities, the differences between the enzymes from particular species being of an essentially minor character[75].

The proteinases in such insects as the cockroach *Periplaneta*[55], the larvae of the blow-flies *Lucilia* and *Calliphora*[13,76], the larva of the clothes moth *Tineola*[75] and that in the saliva of the assassin bug *Platymeris*[77], all resemble mammalian trypsin in possessing neutral or alkaline pH optima. The proteinase extracted from the midgut of the stable fly, *Stomoxys*, has been separated by paper electrophoresis into three protein fractions[78]. This enzyme complex exhibited some marked similarities to mammalian trypsin. The proteinase activity of gut enzymes of *Calliphora*[76], *Periplaneta*, *Musca*[79] and *Stomoxys*[78] have all been shown to approximate to mammalian trypsin in exhibiting activation energies in the range 13,000 to 16,000 cal/mole (*cf.* Sizer[80]). It can thus be supposed that these enzymes are endopeptidases, being able to split the peptide linkages in the main protein chain as well as those of the terminal amino acid residues[81]. It is not known whether the insect enzymes are secreted as inactive precursors, subsequently being activated in the gut. An earlier claim that enterokinase exerted an effect on cockroach proteinases[63] was not confirmed by some subsequent work[75]. There has been no clear-cut demonstration of any enzymes approximating to mammalian pepsin in insects (*cf.* Gilmour[20]).

As in vertebrates the exopeptidase activity of the alimentary canal of several insect species can be attributed to carboxypeptidases, aminopeptidases and dipeptidases[9,55,63,82]. Thus the constituent amino acids of the dietary proteins can be released into the gut lumen as the polypeptide fragments are degraded and the dipeptides eventually hydrolysed.

Insects are apparently unique in the animal kingdom for the ability of certain species to digest and utilize the structural protein, keratin. This very resistant protein is ingested as wool, hair or feathers by chewing lice (Mallophaga), dermestid beetles and larvae of moths of the family Tineidae. The proteinases from the gut of *Tineola* larvae were found to have no digestive action on keratin *in vitro*[83]. As has already been mentioned these enzymes appear to be essentially similar to those of other insects and to mammalian trypsin[75]. The ability of these proteinases to effect digestion of the keratin has been shown to be related to the very unusual reducing conditions found in the midgut in these insects. The oxidation- reduction potential in *Tineola* midgut was shown to be about -200 to 250 mV, in dermestid larvae to be in the range -109 to 230 mV and in the Mallophaga studied[84,85] to be about -200 mV. Under these conditions the disulphide groups of the keratin molecules were reduced to sulphydryl groups, so that the trypsin-like enzymes were thus able to digest the reduced keratin. More exhaustive discussions of this topic are given by Gilmour[20], Hinton[86] and Waterhouse[87].

Active proteinases have been found in most of the insects which have been studied and are absent or very weak only in species adapted to specialized diets such as adult Lepidoptera[14] and the adult blow-fly, *Calliphora*[9]. Thus, as in mammals, most insects are capable of degrading the various ingested proteins to the constituent peptides and amino acids for absorption from the lumen of the alimentary canal.

Absorptive Processes

Although many insects possess complements of enzymes adequate for the complete degradation of proteins to their component amino acids there is

little evidence as to the degree of proteolysis necessary to precede absorption from the alimentary canal. In mammals it is generally assumed, often on *a priori* grounds[88,89], that complete digestion to the free amino acid stage is a prerequisite for intestinal absorption although this view has been strongly challenged[90]. In the assassin bug *Platymeris* the saliva injected into the prey contains an active trypsin-like enzyme, the midgut protease being very weak[77]. In view of the relatively slow rate of trypsin digestion it has been suggested that in the absence of significant midgut protease activity a proportion of the

Table 5.1. The amino acid composition of the haemolymph of *Schistocerca gregaria*[94]

Amino acid	*Concentration* ± S.D.
Glycine	33·2 ± 7·1
Alanine	3·7 ± 2·2
Valine	5·0 ± 2·9
Leucine and isoleucine	2·6 ± 3·8
Proline	4·0 ± 2·5
Tyrosine	2·5 ± 1·5
Serine	34·6 ± 2·3
Threonine	2·3 ± 1·3
Glutamic acid	5·1 ± 2·6
Histidine	1·0 ± 1·1
Glutamine	10·9 ± 1·6

ingested protein material may be absorbed in the form of peptides. There is also good evidence that in the blood-sucking bug, *Rhodnius prolixus*, a certain proportion of the ingested haemoglobin is absorbed into the haemolymph without being digested[91]. The fact that this section deals largely with the absorption of amino acids should, therefore, not be taken as implying that larger protein fragments may not be absorbed from the lumen of the alimentary canal.

Insects are known to possess concentrations of amino acids in the haemolymph which are probably higher than in any other animal group (*cf.* Buck[92]). The absorption of amino acids from the gut lumen in such organisms might, therefore, be expected to present some difficulties due to adverse concentration gradients across the gut wall when the concentration of an amino acid in the haemolymph exceeds that in the lumen of the alimentary canal. Furthermore the absorption of the relatively large monosaccharide molecules from the midgut of the locust *Schistocerca* has been shown to take place largely by facilitated diffusion[33,34], so that the absorption of the small rapidly diffusing amino acid molecules could again be expected to present difficulties in this insect. It should also be borne in mind in this respect that most membranes possessing secretory or transport mechanisms for non-electrolytes appear to be relatively impermeable to passively diffusing molecules[93].

The amino acid content of the haemolymph of adults of the locust *Schistocerca gregaria* is illustrated in *Table 5.1.* It will be seen that this fluid is characterized by high concentrations of glycine and serine, together with glutamine, being similar in this respect to the haemolymph of some other species including that of the larva of the silkworm *Bombyx mori*[95]. In experiments on amino acid absorption in the locust an experimental fluid, in

which the concentrations of the amino acids and other substances were similar to those in the haemolymph, was introduced into the gut lumen[94]. The absorption of ^{14}C-labelled glycine and serine was found to take place most rapidly from the caeca and the ventriculus of the midgut, as was shown for sugar absorption in this species[33,34]. The changes in concentration of total glycine and ^{14}C-labelled glycine in the caeca following injection of the experimental fluid into the gut lumen are illustrated in *Figure 5.12*. These results demonstrated a definite rise in the concentration in the gut

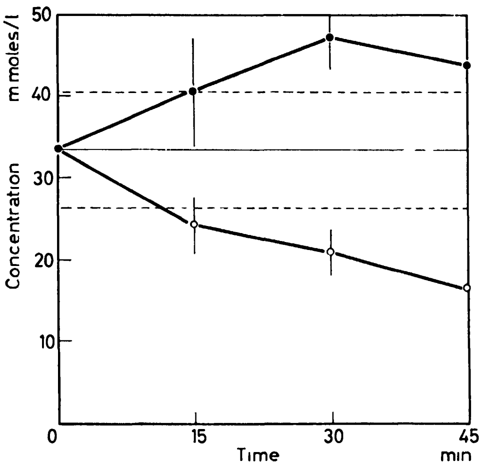


Figure 5.12. Changes in concentration of total glycine (●) and ^{14}C-labelled glycine (○) in the caeca following the injection of the experimental fluid into the gut lumen of the locust *S. gregaria*[94]. The vertical lines and the broken horizontal lines represent the extent of twice the standard error of the mean.

lumen. The radioactive molecules in the caeca tended to fall in concentration, presumably due to mixing with those in the haemolymph. Essentially similar results were obtained with the serine molecules introduced into the lumen of the caeca. Glutamine, which was also introduced into the lumen at a similar level to that in the haemolymph, showed a similar increase in concentration above that in the haemolymph. In experiments using ^{131}I-labelled albumen as a volume indicator it was found that this effect resulted from the removal of water from the lumen of the caeca, a process which occurred more rapidly than the net transfer of the amino acids across the gut wall. It was suggested, therefore, that the absorption of these amino acids was brought about by the diffusion gradient thus created, the transfer of glycine and serine being dependent, in part at least, upon the net movements of water molecules into the haemolymph. The water movements demonstrated in these experiments could have been produced either by an active transport of water or by a passive movement resulting from net ion movements into the haemolymph (*cf.* Robinson[96]). In the absence of a demonstrated accumulation of these amino acids against a concentration

gradient it seems reasonable to suppose that active uptake of serine and glycine forms only a relatively small part of the absorptive mechanism in this insect. The processes responsible for the accumulation of these amino acids in the locust thus seem to be fundamentally different from those taking place in mammals. It has been demonstrated, for example, that steep concentration gradients were established in the absorption of several amino acids from the small intestine of hamsters[97,98].

The amino acids glycine and serine, which occur in high concentration in the haemolymph of such insects as *Schistocerca* and *Bombyx*[95], do not form a substantial proportion of the protein amino acids of plants. In Gramineae, for example, the glycine content is only 2 to 3 per cent of that of arginine and about 4 per cent of the leucine content.[99] It therefore seems probable that many of the amino acids released in the midgut as a result of the digestion of plant proteins are initially at a higher concentration than those in the haemolymph. It could thus be expected that some other amino acids would be absorbed more rapidly than the glycine and serine used in the experiments on *Schistocerca.* The possibility also exists that other amino acids are rapidly absorbed by some specific mechanism such as that demonstrated to occur in the intestine of mammals.

Many species of aphids exhibit a unique form of feeding by tapping with their stylets the nutrient sap stream contained in the host plant's phloem sieve tubes. In the willow aphid, *Tuberolachnus salignus*, the rate of exudation of sap from cut stylets left in the plant tissues was found to approximate to the rate of honeydew production in the intact insect and it has been suggested that, in this species at least, ingestion of the sap is a relatively passive process[100,101]. The nitrogen compounds of the phloem sap were present only as free amino acids and amides, approximately 55 per cent of these ingested compounds being absorbed from the lumen of the gut[50]. Comparison of the amino compounds in the haemolymph and honeydew of the pea aphid, *Acyrthosiphon pisum*, has shown that the total free amino acid content was about the same in both fluids[102]. While some of the 16 amino compounds found in the haemolymph were at a similar level to that in the honeydew, marked differences in the concentrations of some of these compounds were observed (*Figure 5.13*). Thus tyrosine and proline were present at higher levels in the haemolymph; the levels of homoserine and glutamine in the honeydew, on the other hand, exceeded those in the haemolymph. The fact that certain compounds occurred at similar concentrations in the two fluids suggests that their absorption occurred passively. However, it cannot be assumed that the differences in concentration between the haemolymph and the honeydew were due to any differential absorption of these amino acids from the lumen of the alimentary canal. For example, such differences could be the result of a rapid metabolism of the absorbed compounds or to some excretory processes associated with the alimentary canal in this insect. It is to be hoped that some future work will help to establish the relative importance of these various processes in maintaining the concentration differences of these amino acids across some portion of the gut wall in aphids.

In the larvae of the mosquito *Aedes aegypti* deposits of glycogen were demonstrated to accumulate in scattered cells in the caeca and in the pyloric half of the midgut following the ingestion, by starved individuals, of casein,

alanine and glutamic acid[53]. It is not known what proportion of the total absorbed amino acids contributed to the appearance of the glycogen in these specialized cells. If the glycogen was, in fact, derived from amino acids which entered the cells directly from the gut lumen then this could suggest that absorption was effected by some sort of facilitated diffusion mechanism. Thus a rapid conversion of the amino acids to the polysaccharide may have resulted in a steep concentration gradient into the cells' cytoplasm. Such a mechanism would presumably only operate for amino acids such as alanine

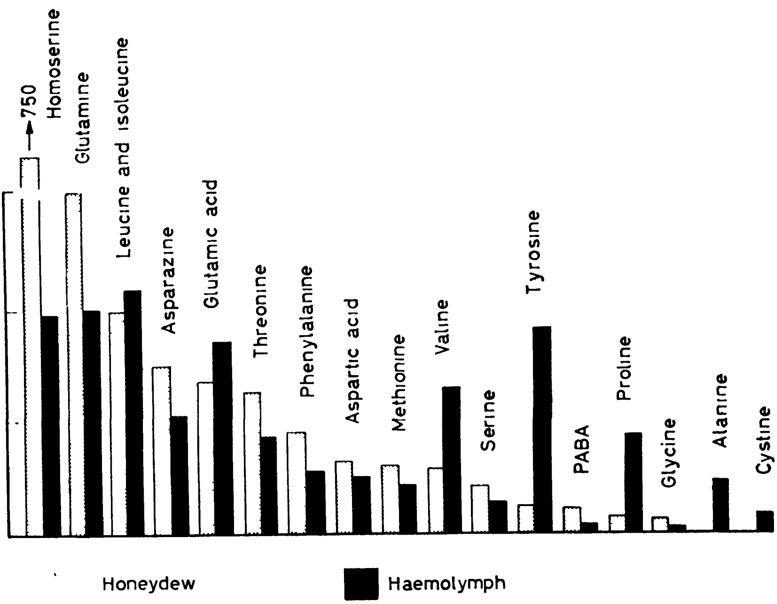


Figure 5.13. Concentration of amino acids in the haemolymph and honey dew of the pea aphid *Acyrthosiphon pisum* (data from Auclair[102])

and glutamic acid which could be readily incorporated into the carbohydrate metabolism of the cells via the tricarboyxlic acid cycle (*cf.* Treherne[41]).

In the blood-sucking bug *Rhodnius prolixus* most of the ingested haemoglobin was found to be broken down in the gut to protohaemoglobin which was shown to be excreted unchanged[91]. However, a certain amount of katahaemoglobin was also found to accumulate in the haemolymph, suggesting that a small proportion of the haemoglobin was absorbed without prior digestion. The absorption of the unchanged molecule was clearly shown not to depend upon any damage caused to the gut by excessive distension following a blood meal. The louse *Pediculus* and the tick *Ixodes* were also similar to *Rhodnius* in absorbing a proportion of the ingested haemoglobin in an unchanged form, although in some other blood-sucking insects, including the flea *Nosopsyllus* and two species of mosquitoes, there appeared to be no appreciable absorption of the haemoglobin. The distribution of free iron and pigments in *Rhodnius* suggested that an appreciable breakdown of haemoglobin occurred in the

epithelial cells of the midgut. It was assumed that the haemoglobin diffused into the midgut cells and was there transformed into an altered haematin, a verdohaem pigment and the blue-green pigment, biliverdin. The biliverdin was found to be eventually discharged from the cells of the midgut into the lumen. These observations demonstrate an aspect, little emphasized, of the absorptive function of the midgut cells in participating in the excretion of metabolic waste products. There was no demonstrable accumulation of these pigments in the midgut cells of some other blood-sucking species including the bed-bug *Cimex*, the flea *Nosopsyllus* or in the mosquitoes *Aedes* and *Anopheles*[91].

The absorption of amino acids and polypeptides may also be involved in another example of an excretory function of the midgut epithelial cells. In the larvae of *Tineola* the digestion of wool impregnated with various metallic and non-metallic elements was found to result in the formation of insoluble sulphides due to the liberation of hydrogen sulphide from reduced and hydrolysed cystine[84]. A proportion of some of the insoluble sulphides were found, nevertheless, to have accumulated in the goblet cells of the midgut. It was suggested, on the basis of the work of Neuberg and Mandl[103], that the polypeptides and amino acids produced during digestion formed a finely dispersed colloidal solution with part of the sulphides which were then absorbed into the cells in this form. It was visualized that the sulphides, polypeptides and amino acids were first taken up by the columnar cells, which had a large surface area, and were then passed without accumulation into the goblet cells from which the sulphides were discharged into the cavities. It was pointed out that in the anterior and posterior portions of the midgut the columnar cells were not in contact with the goblet cells which were shown to accumulate the sulphides less readily. The accumulated sulphides in the cavities of the goblet cells were eliminated during moulting when the entire midgut epithelium is renovated[84].

THE ABSORPTION OF WATER AND INORGANIC IONS

Different species from the various insect groups differ very widely in the composition and water content of their diet. Thus the flour moth *Ephestia kuehniella* can live on food containing only 1 per cent of water[104], while such insects as aphids, butterflies and blood-sucking species feed on diets consisting very largely of water. Among aquatic insects the larvae of *Aedes aegypti* can ingest distilled water while the related species *A. detritus* and other euryhaline Diptera can drink the highly saline waters in which they normally live[105,106]. As would be expected, therefore, insects have evolved a great variety of physiological and structural adaptations for the absorption of inorganic ions and water molecules, from the lumen of the alimentary canal.

Absorption in the Foregut

As with monosaccharides and lipids the foregut of insects does not appear to be involved in the absorption of significant amounts of ingested water and salts. The crop of the cockroach when used as an osmometer has been found to be virtually impermeable to water[4,5]. A relatively high degree of

impermeability can also be attributed to the crop wall of *Culex pipiens* for an aqueous solution placed in an isolated crop in this species showed no signs of drying up after several days[107]. Similarly the crops of the cockroach *Periplaneta americana* and of the locust *Schistocerca gregaria* must be relatively impermeable to water for no concentration of dye solutions could be demonstrated in the foregut of these species[31,33].

Absorption in the Midgut

Although there have been few unequivocal demonstrations of water uptake it is generally assumed that various regions of the midgut represent major sites of water absorption in most insects. Wigglesworth[108] clearly showed that in larvae of the mosquito *Aedes aegypti*, reared in fresh water, particles of the dye trypan blue were rapidly concentrated in the midgut caeca. These larvae were, however, unable to take up water from the lumen of the caeca when they were placed in a 1 per cent solution of sodium chloride, presumably indicating that some osmotic uptake was involved[109]. The concentration of dyestuffs in the midgut of larvae of the blow-fly *Lucilia* was found to take place largely in the middle region of this part of the alimentary canal[13]. It is perhaps significant that in this insect the acidity of the midgut brings about a coagulation of the proteins in the gut lumen. It seems possible that this effect, by lowering the osmotic pressure of the midgut contents, may well facilitate the absorption of water from this portion of the alimentary canal. In the locust *Schistocerca*, a relatively rapid absorption of water can be inferred from the rate of concentration of ^{131}I-labelled albumen in the region of the midgut caeca[94].

In mosquito larvae there is evidence that much of the fluid present in the midgut was not, in fact, ingested through the mouth. Wigglesworth[108] has pointed out that in *Aedes aegypti* anti-peristaltic waves travelled along the midgut, thus tending to carry fluid to the caeca. Using dyes Ramsay[105] also showed that a significant amount of the midgut fluid was probably derived from fluid excreted by the Malpighian tubules. The possibility also exists that some of the fluid reached the caeca from the haemolymph via the wall of the midgut itself. The relatively free permeability to water of this portion of the gut was demonstrated in experiments in which midgut was shown to become rapidly distended following ingestion of 2·0M sucrose solution[105]. There is then, apparently, in this species an internal circulation of water from haemolymph → midgut → caeca → haemolymph. However, a forward circulation of fluid from the tubules into the midgut does not apparently take place in the alimentary canal of the stick insect *Dixippus morosus*[1].

A good deal of information about the nature of the processes involved in the transfer of water and salts between the gut lumen and the haemolymph has emerged from some very detailed studies of the osmotic regulation in some aquatic insects. The osmotic conditions in the various parts of the alimentary canals of larvae of the fresh-water mosquito *Aedes aegypti* and the saline species *A. detritus* for individuals maintained in different media are illustrated in *Figure 5.14*. These data show that in both species the caecal fluid was invariably hypertonic to the haemolymph in all media which, taken with the previous evidence of water uptake, must indicate that the

cells of the caeca are capable of appreciable osmotic work. On the other hand, the midgut fluids were, in both species, approximately isotonic to the haemolymph. The changes in the sodium and potassium content of this fluid, which was presumed to be largely derived from the Malpighian

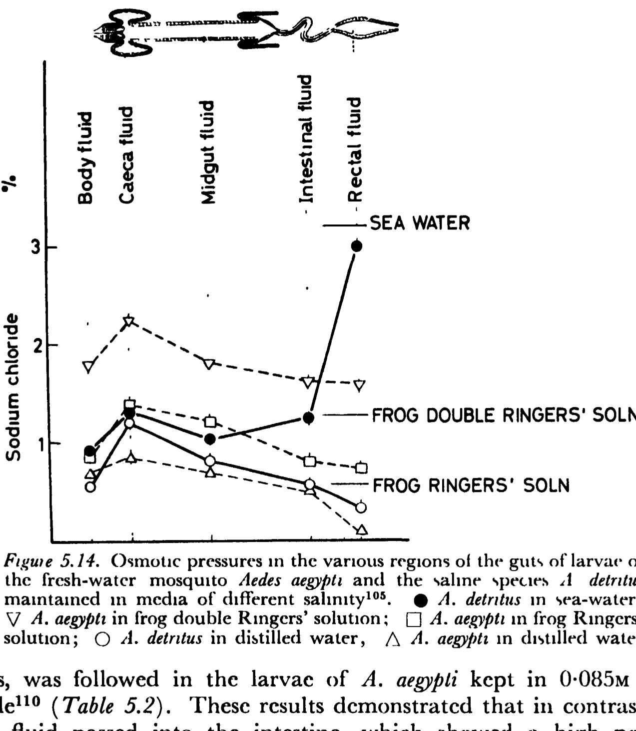


Figure 5.14. Osmotic pressures in the various regions of the guts of larvae of the fresh-water mosquito *Aedes aegypti* and the saline species *A. detritus* maintained in media of different salinity[105]. ● *A. detritus* in sea-water, ▽ *A. aegypti* in frog double Ringers' solution; □ *A. aegypti* in frog Ringers' solution; ○ *A. detritus* in distilled water, △ *A. aegypti* in distilled water

tubules, was followed in the larvae of *A. aegypti* kept in 0·085M sodium chloride[110] (*Table 5.2*). These results demonstrated that in contrast to the tubule fluid passed into the intestine, which showed a high potassium concentration, the fluid discharged into the midgut showed a substantial

Table 5.2. Effect of the external ionic concentration on the composition (values expressed in mmoles/l.) of the fluid from various parts of the alimentary canal of *Aedes aegypti*[110, 111]

Fluid	*Distilled water*		0·085M NaCl		0·085M KCl	
	Na	K	Na	K	Na	K
Haemolymph	87·0	3·1	113 0	—	—	5·7
Midgut fluid	—	—	165·0	26·0	—	—
Proximal tube fluid	25·1	—	—	—	—	—
Intestinal fluid	24·0	88·0	71·0	90·0	23·0	138·0
Rectal fluid	4·0	25·0	100·0	18·0	14·0	90·0

return to a sodium/potassium ratio characteristic of the haemolymph. It could be tentatively supposed that, in these circumstances, the ionic composition of the midgut fluid had been partly achieved by a passive diffusion of the potassium ions from the midgut lumen due to the steep concentration gradient into the haemolymph.

In the aquatic larvae of the alder fly *Sialis lutaria* the alimentary canal is of considerable importance in its function of absorbing ions from the dilute external solutions[112]. The relationship between the sodium and chloride concentrations of the ingested external solution and the haemolymph is illustrated in *Figure 5.15.* At concentrations exceeding about 130 mmoles/l. both the ions attained a similar level to that of the external medium; at lower concentrations the sodium and, to a lesser extent, the chloride ions were maintained at a considerably higher level than in the ingested solution. The uptake of chloride ions via the alimentary canal was, however, found to be approximately proportional to the chloride concentration of the ingested external solution (*Figure 5.16*). The potential difference across the midgut wall of *Sialis* averaged about 18·0 mV, the haemolymph being positive to the midgut contents[113]. The measurements were made with sodium chloride-filled electrodes so that the figure for the average potential difference across the midgut wall will also include the junction potential which would be expected to amount to as much as 3 mV[114]. Using the figure of 18·0 mV and the values for the concentrations of chloride in the haemolymph the equilibrium concentration C of the ion in the midgut lumen was calculated from the relationship:

$$C_{\text{midgut}} = C_{\text{haemolymph}} \exp \left[\frac{zF}{RT}(E_0 - E_i)\right]^{-1}$$

where z — valency of the ion, F = Faraday's constant, T = absolute temperature and $E_0 - E_i$ = the potential difference. The equilibrium chloride concentration was found to be 15·2 mmoles/l., thus indicating that the demonstrated uptake of chloride could be explained in terms of a passive diffusion of the ions across the midgut wall. The absorption of these ions from dilute solutions was not studied so that the mechanism of chloride uptake below the concentration of 15 mmoles/l. is not known.

The values for the uptake of sodium ions, from a 0·085M solution of sodium chloride, indicated that the rate of absorption was approximately similar to that of the chloride ions[113]. However, a potential difference of about 18·0 mV at this concentration would mean that the apparent equilibrium concentration of sodium ions in the gut would be approximately 218 mmoles/l. This implies that the uptake from the 0·085M sodium chloride solution must have involved an active process associated with the cells of the midgut wall. Thus despite the similarity in the rates of absorption of sodium and chloride ions from the midgut in *Sialis* very different mechanisms were apparently involved in the two processes. Preliminary experiments on the uptake of potassium appeared to show that the absorption of these ions occurred less rapidly than that of chloride. As has already been concluded in the case of *A. aegypti*[110] the uptake of potassium from the midgut at the high concentrations used in these experiments could have been due to a passive diffusion, although the possibility of a relatively slow active absorption cannot be eliminated[113].

The gut of larvae of *Sialis* was clearly implicated as a major site of water absorption in experiments concerned with the gain in weight of individuals from which large amounts of the haemolymph had been removed[115]. The entry of water via the gut under these circumstances was found to exceed

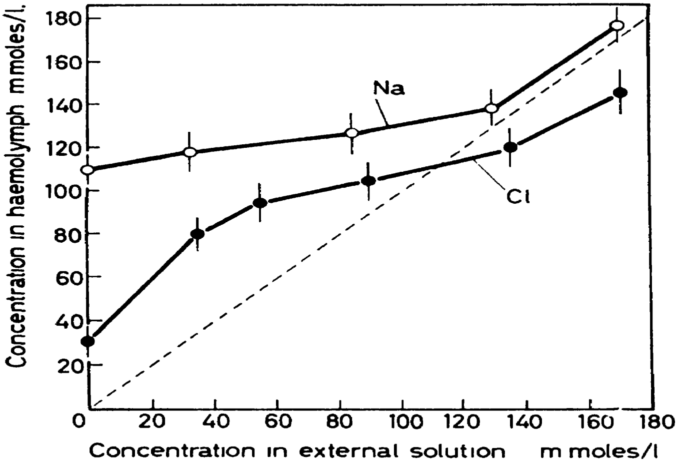


Figure 5.15. Relationship between the sodium and chloride concentrations of the haemolymph and of the ingested external solution in larvae of *Sialis lutaria*[113]

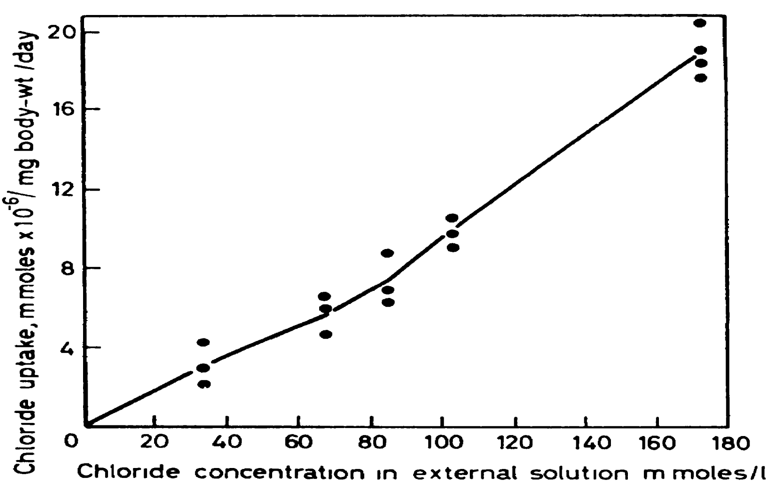


Figure 5.16. Chloride uptake by the gut of *Sialis lutaria* from solutions of varying salinity[113]

the osmotic uptake through the general surface of the cuticle by about three times for larvae kept in tap-water. However larvae with reduced haemolymph volumes on ingestion of isotonic mannitol showed no significant increase in weight during the experimental period, a fact which clearly indicated that the net absorption of water from dilute solutions through the gut was due to osmotic uptake. The uptake of water from various ingested isotonic salt solutions was found to depend upon the nature of the cation present[113]. There was no significant gain in weight in haemolymph-deficient

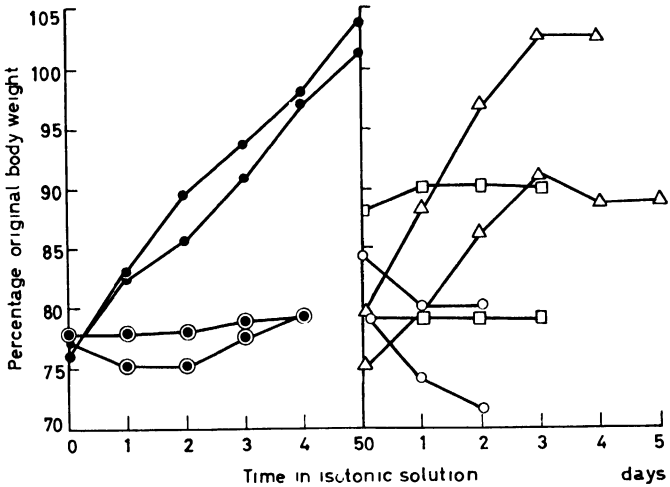


Figure 5.17. Uptake of water by *Sialis* larvae with reduced haemolymph volume in various isotonic solutions[113]. ● larvae in NaCl; ◎ same with necks ligatured; △ in $NaHCO_3$; □ in $CaCl_2$; ○ in KCl

larvae kept in isotonic potassium chloride or calcium chloride (*Figure 5.17*). Similar individuals which were maintained in isotonic sodium chloride or sodium bicarbonate, on the other hand, showed an ability to gain water rapidly, suggesting that in these individuals water transport was associated with the simultaneous absorption of salts. In this species, in view of the demonstrated uptake of chloride from solutions of potassium chloride and the uptake of water from isotonic sodium bicarbonate, it is obvious that the process of water absorption is linked in some way with the active uptake of sodium ions by the gut wall. As Shaw[113] points out, since the uptake of chloride ions apparently occurs positively and at the same rate as that of sodium the total absorption from the gut lumen is equivalent to absorption of a solution of sodium chloride which is approximately isotonic with the haemolymph. Thus water can be absorbed from the gut of *Sialis* by two mechanisms. It can be taken up osmotically through the gut wall from dilute solutions and, in addition, in the presence of sodium ions may be transported across the wall of the gut by a process which is probably linked to the active absorption of these ions. The site of water absorption in the alimentary canal of the larval *Sialis* has not been precisely identified, but by analogy with the

other insect species which have been studied may tentatively be supposed to be located in some portion of the midgut.

A certain amount of information concerning the physiological processes involved in the absorption of copper and iron has been inferred from histochemical studies demonstrating the distribution of these substances in the cells of the midgut of some species of Diptera[54,116,117]. In the larva of the blow-fly *Lucilia cuprina*, for example, a distribution of ferric iron and a smaller amount of ferrous iron has been detected in a short band of cells at the posterior end of the middle region of the midgut (*Figure 5.11*). An increase in the level of copper in the larval diet resulted in the additional appearance of a mosaic of cells containing ferric and ferrous iron in a zone anterior to the original short band of cells (*Figure 5.11*, zone II)[54]. The correlation of iron accumulation with copper metabolism in the midgut cells of this insect appears to parallel the vertebrate condition where a similar effect of copper on the uptake of iron has been noted[118]. The presence of the iron-accumulating cells in an acid region of the midgut may be of some significance for under these conditions it could be expected that the ferric complexes and ferric hydroxide micelles are broken down to free ferric ions (*cf.* Granick[119]). Thus in insects it is possible that, as in mammals, the presence of substances such as ascorbic acid, glutathione or cysteine results in a reduction to the ferrous state in which form it can be absorbed[120].

Absorption in the Hind Gut

The fluid elaborated by the Malpighian tubules together with that discharged by the midgut are subjected to the further absorptive processes associated with the intestinal region of the hind gut. The ability of this region of the alimentary canal to absorb water was demonstrated by Wigglesworth[121] who observed that in a wide variety of insects there was a progressive drying of the gut contents in their passage through this portion of the hind gut. In the larvae of *Aedes aegypti* and *A. detritus* the intestinal fluid, which was supposed to be almost exclusively derived from the Malpighian tubules[111], was shown to be isotonic or slightly hypertonic to the haemolymph (*Figure 5.14*)[105]. The high potassium/sodium ratio characteristic of the Malpighian tubules appeared to be maintained in the intestinal fluid of *A. aegypti*[110] suggesting that in this species the intestine was not effective in altering significantly the composition of the tubule fluid in its passage towards the rectum. In the terrestrial insect *Dixippus morosus* a direct comparison of the urine discharged from the Malpighian tubules and of the intestinal fluid also showed that the intestine did not alter appreciably either the osmotic pressure or the ionic composition of the fluid in the lumen[1]. There was, nevertheless, a demonstrated reduction in the volume of ligatured segments of this portion of the hind gut, clearly showing that absorption was occurring without appreciable alteration of the composition of the intestinal contents. Such a mechanism is difficult to visualize on the basis of fluid movements caused by a hydrostatic pressure gradient produced by the circular muscles of the intestine, for under these circumstances it would not be expected that the concentration differences could be maintained across the gut wall. It is perhaps conceivable that the absorption

is effected by some mechanism of the type demonstrated by Shaw[113] in *Sialis* in which the active sodium uptake was linked to the net water movements to produce a transfer equivalent to the movement of an isotonic sodium chloride solution. According to this hypothesis it would, in addition, be necessary to postulate a movement of potassium ions down a steep concentration gradient which would also parallel the rate of transfer of the sodium and chloride ions across the wall of the intestine.

The contents of the anterior portion of the hind gut are eventually discharged into the lumen of the rectum where they are finally modified by further absorptive processes associated with the rectal epithelium before being discharged as urine or dry faecal pellets. The ability of this terminal portion of the insect hind gut to effect an appreciable absorption of water from the rectal contents was demonstrated in terrestrial insects from a variety of orders by Wigglesworth[121]. In particular it was suggested that the well developed groups of cells, the rectal pads or papillae, were largely responsible for this process.

Several insect species have been shown to produce a rectal fluid which is markedly hypertonic to the haemolymph. Among euryhaline Diptera the larvae of *Aedes detritus* (*Figure 5.14*)[105], *Ephydra riparia* and *Coelopa frigida*[106] are known to produce extremely hypertonic rectal fluids. Similarly among terrestrial insects *Dixippus morosus*[1], *Calliphora erythrocephala* and *Schistocerca gregaria*[122] also have rectal fluids which have been shown to be hypertonic to the haemolymph. Certain fresh-water insects on the other hand are able to elaborate rectal fluids which are markedly hypotonic to the haemolymph. This group includes larvae of *A. aegypti*, *A. detritus* in distilled water (*Figure 5.14*)[105] and *Sialis lutaria*[113].

The production of a hypotonic fluid in the rectum of larvae of *A. aegypti* maintained in distilled water has been shown to be accompanied by a dramatic fall in the concentrations of sodium and potassium (*Table 5.2*) which, it was presumed, resulted from an absorption of both these ions into the haemolymph[110]. There is, however, no direct evidence of the extent of any dilution of the rectal contents by secretion of water into the rectum, although in the face of the manifest ability of this species to conserve its ion content (*cf.* Treherne[123]) it is perhaps reasonable to suppose that this factor may be relatively insignificant. Thus the uptake of sodium ions from the lumen of the rectum must have occurred against a concentration gradient, although in the absence of measurements on electrical potential it is impossible to establish whether this was due to an active transport of these ions. The movement of potassium ions, on the other hand, took place from a high concentration to a relatively low one in the haemolymph so that in this case it may not be necessary to postulate the existence of any active absorptive processes. The uptake of these ions from the rectal fluid thus completes the circulation of potassium within the insect's body which, as Ramsay points out, appears to take place irrespective of the ionic composition of the external medium (*Figure 5.18*).

In contrast to the mosquito larva the rectal fluid of the larvae of *Sialis lutaria* in fresh-water was only barely hypotonic, the osmotic pressure being at least two-thirds that of the haemolymph[113]. The composition of the fluid in the rectum of this insect was remarkable for the apparent absence of

chloride ions, the anion side being made up almost entirely of bicarbonate (*Table 5.3*). The sodium and potassium in the rectal fluid only accounted for a very small proportion of the cation fraction most of which was found to consist of ammonium ions. The potential difference across the rectal wall in *Sialis* was found to average 24 mV, the haemolymph, in contrast to the measurements made on the midgut, being negative to the rectal fluid. The apparent equilibrium concentration of the bicarbonate in the rectum,

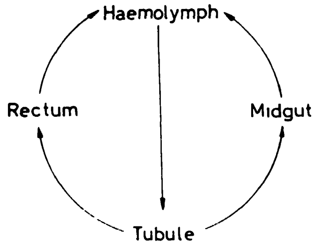


Figure 5.18

calculated on the basis of the observed potential difference, approached the value for this ion determined by chemical analysis. The measured concentration of ammonium ions in the rectal fluid, on the other hand, greatly exceeded the apparent equilibrium concentration, while the levels of sodium and chloride were lower than would be expected for the passive distribution of these ions in accordance with the observed potential difference across the

Table 5.3. The composition (values expressed in mmoles/l.) of the haemolymph and rectal fluid of *Sialis lutaria*[113]

Substance	*Haemolymph*	*Rectal fluid*
Sodium	109	12
Potassium	5	4
Calcium	7·5	—
Magnesium	19	—
Chloride	31	0
Bicarbonate	15	91
Ammonia	—	100

rectal wall. As Shaw has pointed out these results are not inconsistent with the hypothesis that the bicarbonate and sodium ions distribute themselves on either side of the rectal wall according to a Donnan equilibrium, the non-penetrating ions being ammonia in the lumen of the rectum, and chloride and other anions in the haemolymph. However, the possibility cannot be eliminated on the basis of these results that some active absorption of sodium ions occurs in the rectum of *Sialis* larvae. The potassium concentration of the fluid discharged into the rectum was found to lie between 19 and 36 mmoles/l. as against 4 mmoles/l. in the fully elaborated rectal fluid. Thus, as in the mosquito larva, potassium is clearly absorbed from the lumen of the rectum. Similarly the measured potential difference is such that it is not necessary to suppose that any active processes are associated with the uptake of this cation. The larvae of *Drosophila melanogaster* may also be

similar to *Sialis* in possessing a rectal fluid containing an appreciable proportion of an organic anion which, it is suggested, is not absorbed from the lumen of the rectum in larvae maintained in a high potassium chloride medium[124].

In the aquatic larvae of *Chironomous* and *Limnophilus flavicornis* the rectum is effective in removing chloride from the rectal fluid of individuals in dilute external media, although it has not been demonstrated whether this contributes to the establishment of an osmotic gradient across the rectal wall[125].

In the terrestrial insect *Dixippus morosus* the conservation of the body water is of major importance and the rectal fluid was found to be markedly hypertonic, the osmotic pressure being equivalent to a solution of about 390 mmoles/l. as against 159 mmoles/l. in the haemolymph[1]. This high osmotic pressure, which has been presumed to be the result of withdrawal of water from the lumen of the rectum, was largely accounted for by the high potassium content (327 mmoles/l.) derived from the fluid excreted by the Malpighian tubules. In view of the ability of this insect to conserve its body potassium, it is necessary to postulate an appreciable reabsorption of potassium in the hind gut. As has already been mentioned a certain amount of absorption takes place in the anterior part of the hind gut. The remainder of the uptake of the potassium excreted by the Malpighian tubules thus occurs in the rectum, where it is possible that the ions are absorbed by diffusion down the steep concentration gradient maintained across the rectal wall. In this insect then, unlike the fresh-water species previously described, the circulations of potassium and water in the excretory system and the rectum appear to parallel one another.

The apparent active absorption of water from the rectum of terrestrial insects was confirmed in some recent experiments on the locust *Schistocerca gregaria*, in which the withdrawal of water and the simultaneous establishment of an osmotic gradient across the rectal wall was demonstrated from pure sugar solutions[122]. A dependence of the net movement of water on the osmotic gradient was shown in experiments in which the rate of uptake of water from the lumen was found to be approximately linearly related to the initial osmotic pressure difference across the rectal epithelium. Such an effect resembles that which would be expected with a passive diffusion of water molecules except that the system came into equilibrium with a relatively large osmotic gradient of 0·6 osmoles across the rectal wall. These facts have been interpreted as representing evidence for the existence of a passive flux component which is superimposed upon an active mechanism capable of absorbing water from the rectum at a constant rate of about 17 μl./hour, at 18 to 22°C, irrespective of the osmotic gradient. There is, however, no indication as to the nature of the system capable of producing the active transfer of water molecules across the rectal epithelium. Phillips[122] has clearly shown that such factors as electro-osmosis, the efflux of hydrated ions from the rectum and hydrostatic pressure gradients are unlikely to account for the observed uptake of water from the rectal fluid. The energy output of the rectum is, however, probably adequate to supply the minimum theoretical energy of 0·006 cal/hour required for water transport.

The measured uptake of water from the rectum of the blow-fly *Calliphora erythrocephala* which, unlike the locust, normally excretes a watery urine,

indicated that the physiological processes involved were essentially similar to those occurring in the rectum of *Schistocerca*[122].

The active secretion of water in the rectum of some terrestrial species apparently distinguishes the insects from the vertebrate animals which have been investigated where the uptake of water in the intestine appears to be very largely a passive process resulting from the relatively rapid absorption of sodium ions and glucose molecules[126,127]. There is, in fact, very little evidence to support the hypothesis of an active transport of water in other living organisms (*cf.* Robinson[128]) and it is even conceivable that the insects and, perhaps, other arthropods may be unique in this respect.

Some further experiments with *Schistocerca* have shown that the rectum can absorb sodium, potassium and chloride ions against steep concentration gradients[122]. As in *Sialis*[113] the lumen of the rectum is positive to the haemolymph, the measured potential difference of 15 to 30 mV being of the same order as that for the aquatic insect. The magnitude of the potential difference across the rectal epithelium implies that the chloride ions must be actively transported into the haemolymph. It is conceivable that this active reabsorption of chloride may be associated with those species which, like the locust, maintain a high concentration of this anion in the haemolymph. As Phillips points out, it is also probable that sodium and potassium may be actively transported for the observed potential gradient was only about 20 to 30 per cent of that required to maintain the concentration gradients of the ions. In the blow-fly, on the other hand, the fluid entering the rectum is almost devoid of chloride, so that appreciable transport of this anion cannot occur in this species[122].

The cuticular intima lining the rectum of the locust has been found to act as a molecular sieve restricting the diffusion of relatively large solute molecules and ions[122]. This membrane was found, for example, to be 10 to 100 times more permeable to water than to potassium and sodium chlorides, while the rate of diffusion of a disaccharide such as trehalose was found to be only 0·3 per cent of that of sodium chloride. Permeability experiments with various dyes indicated that a large proportion of any water-filled pores must have effective radii of the order of approximately 7 Å. These data suggest that the cuticular intima, which is very different to that lining the foregut, is specialized to allow the reabsorption of relatively small, physiologically important molecules such as water, various salts and amino acids. Similarly this molecular sieve is likely to be effective in excluding large organic molecules which are eventually passed out of the rectum in the faeces.

GENERAL CONSIDERATIONS

The picture which emerges from the evidence so far available indicates that many of the processes involved in the absorption of nutrient substances from the alimentary canal in insects appear to be very different from the equivalent processes in mammals. The absorption of monosaccharides from the midgut of the locust, for example, was shown to be effected probably by a facilitated diffusion mechanism involving the conversion and the subsequent accumulation in the haemolymph of the disaccharide trehalose. There was no evidence of any appreciable active absorption of monosaccharides from the gut

lumen in this species. The uptake of glucose in the mammalian intestine, on the other hand, can proceed against a concentration gradient and is presumed to be the result of some active mechanism[46,129]. There is, however, no *a priori* reason for supposing that active processes for sugar absorption are absent in all insects. Some dipterous larvae have been shown to possess a haemolymph containing relatively high concentrations of glucose, so that absorption of this monosaccharide cannot be achieved by facilitated diffusion and it is possible that in these forms some active mechanisms analogous to those in mammals may exist.

Similarly the few experiments which have been carried out on the absorption of amino acids have failed to demonstrate the establishment of concentration gradients across the gut wall as has been demonstrated in the intestine of mammals[91,98]. In the locust the uptake of glycine and serine, and also glutamine, appeared to be effected by diffusion down the concentration gradients caused by the relatively rapid removal of water from the gut lumen. These compounds, although convenient for experimental study, are not common constituents of plant proteins and the possibility of the active transfer of other amino acids cannot be eliminated.

The absorption of fats in insects, as in mammals, has been the subject of much controversy and it is only relatively recently that unequivocal demonstrations of the sites of absorption have been made. Nothing is known about the nature of the absorptive mechanism involved except that in the very specialized case of the uptake of the products of wax digestion in the wax moth some sort of intermediate phosphorylation may be involved. The absorption of these compounds thus appears to be similar in this respect to the processes involved in lipid absorption in mammals (*cf.* Frazer[130]).

The uptake of inorganic ions in the midgut has been clearly shown in one insect species to involve an active transport of sodium into the haemolymph. The movements of potassium and chloride ions in this region of the alimentary canal appear to be essentially passive processes in the species which have been investigated. In the rectum, which has long been recognized as a site for the possible uptake of water and salts, the absorption appears to be characterized by the presence of mechanisms capable of effecting an active transport of sodium ions. As in the midgut, the circulation of potassium in this terminal portion of the hind gut does not, with the possible exception of the locust, in general require the postulation of any active mechanisms. In the case of the locust rectum it is also conceivable that chloride may be absorbed actively, possibly by a separate mechanism from that involved in the accumulation of sodium into the haemolymph. The various insects which have been studied thus appear to be similar to the vertebrates in which active mechanisms have been identified as being responsible for the uptake of both sodium and chloride in the ileum[126]. The uptake of iron in the midgut of various species of Diptera may also bear some resemblance to the similar processes in the mammalian intestine.

The available experimental evidence indicates that the demonstrated uptake of water from the rectum in terrestrial and euryhaline aquatic insects is probably effected by some active mechanism which is superimposed upon the passive movements of water molecules across the rectal wall. This active movement of water into the haemolymph appears to be fundamentally

different from the water absorption in the mammalian ileum which has been interpreted as being a passive process depending on the gradients of water activity and on the rate of absorption of solute[126]. At the moment there is little evidence to support the hypothesis of active transport of water in other animal groups[128]. It is even conceivable that arthropods may be unique in this respect.

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6

SOME NEURAL CORRELATES OF PERCEPTION

GABRIEL HORN

INTRODUCTION

IN ORDER that an object may be perceived, it must fall within the sensory field of the organism and evoke activity in some sort of detecting apparatus. The information contained in the stimulus must be coded for transmission to such places in the nervous system where it can be interpreted according to the context of information arriving along other sensory channels, and in the light of information gained from past experiences. These interactions are essential if perception is to mean anything more than the experience of isolated sounds, pressures and patches of light.

Because human beings are able to communicate in a unique way, they are ideal subjects for experiments that seek to establish correlations between neural events and perceptual experiences. These correlations can be made only indirectly and incompletely in animals. Since the sort of experiments that can be done on humans is clearly limited, it is often necessary to use information gained from animal studies as a basis for interpreting the results of psycho-physical investigations on human subjects.

The dangers of extrapolating in this way are too obvious to mention, but there may well be a tendency to overstate them. No one would seriously question the fact that the nervous system of man is more complex than that of other animals, but where it has been possible to compare the basic properties and organization of the human brain with that of certain other animals, the differences have not been conspicuous. In the present state of knowledge, therefore, extrapolation from the behaviour of non-human to human brains is not unwarranted, and is likely to be useful, in so far as it leads to the formulation of hypotheses of the kind that may be tested on human subjects.

In this review I shall attempt to describe some of the mechanisms whereby a stimulus falling on a sense receptor leads to the generation of nerve impulses, to discuss the ways in which various attributes of the stimulus are coded and transmitted in the nervous system, to indicate how this transmission appears to be controlled, and finally, to summarize what little is known about the neural basis of sensory integration and memory. Since the nerve impulse is the fundamental unit of communication within the nervous system, I shall first outline its properties and describe how signals are passed from one nerve cell to another.

It is outside the scope of this short review to give an exhaustive and critical account of recent advances in the expanding field which it attempts

to encompass. It is necessary to be highly selective, and to write about work that seems, at least to the author, to be particularly relevant and interesting, and to graft the information so derived on to the general body of anatomical and physiological knowledge of the nervous system that has accumulated over the years.

INTRA- AND INTER-NEURONAL TRANSMISSION

In 1939 Hodgkin and Huxley[1], investigating the electrical events that accompany the nerve impulse, succeeded in introducing a fine open-ended glass capillary tube into the giant axon of the squid *Loligo forbesi*. These axons are relatively large, having diameters of about 0·5 mm. The capillary tube was filled with sea-water which was connected through a length of silver wire to an amplifier and thence to a cathode ray oscilloscope. Potential changes in this recording electrode were measured with reference to a second electrode in the sea-water outside the axon. It was found that in the resting, non-conducting axon, there was a potential difference across the membrane amounting to about -50 mV, the inside being negative in relation to the outside. This resting, polarized state underwent a marked change when the fibre was stimulated. As the impulse passed beneath the electrode the charge across the membrane was reversed and the potential difference amounted to about $+40$ mV. Thus a potential change (the action potential) of 90 mV accompanied the nerve impulse.

In a fluid, or semi-fluid system such as living matter, changes in electrical potential are brought about through the movement of ions; so any adequate account of the nature of the nerve impulse must be expressed in terms of ionic movement. This has now been done and the hypothesis so derived has been subjected to rigorous quantitative analysis[2,3]. Although the bulk of experimental work has been done on the unmyelinated squid axon, there is no reason to doubt that the general conclusions are also applicable to myelinated peripheral nerve fibres of vertebrates[4,5]. The work may be summarized, with considerable over-simplification, as follows.

The axon is bounded by a membrane which, when the fibre is at rest, can be regarded, to a first approximation, as being freely permeable to potassium ions, but impermeable to sodium ions. There is a higher concentration of potassium inside the fibre than outside, while the reverse holds true for sodium. If we assume that the internal and external potassium ions move freely and at random, some will collide with the membrane and, when they encounter a hole, will pass through to the opposite side. There are roughly 10 times as many potassium ions inside the axon as in the external medium, so that the inner surface of the membrane will be hit by potassium ions 10 times as often, on the average, as will the outer surface. The result will be that about 10 times as many potassium ions will pass out of the fibre as pass in. As each positively charged potassium ion passes out, it leaves behind an anion whose negative charge goes un-neutralized. The inflowing potassium ions are numerically inadequate to restore electrical neutrality, so that a negative charge begins to build up inside the membrane as the number of free anions increases. This increasing negative charge pulls back some of the positively charged potassium ions and a stage is

reached when the negative charge generated by the free anions inside the axoplasm draws back as many positively charged potassium ions as leave. When this stage is reached the system is in equilibrium and the potential difference across the membrane is known as the potassium equilibrium potential. Its magnitude is close to, though not identical with, the resting potential.

When a nerve fibre conducts an impulse, the properties of its membrane change. For a brief period, rather less than a millisecond, the active zone of membrane becomes highly permeable to sodium ions. These now pass through the membrane and tend to establish a sodium equilibrium potential, which is opposite in sign to the potassium potential, since sodium is in excess outside. The active membrane is so highly permeable to sodium that this equilibrium potential is approached quite closely at the height of the action potential. After a short time interval, however, which is of the order of 1 msec or less, the sodium permeability is abolished and the potential rapidly returns toward the potassium equilibrium potential. The electrical changes that accompany these rapid phases of ionic movement constitute the action potential. The net result of these fluxes is an exchange of a minute fraction of the internal potassium for external sodium. If the movement of the two ions occurred concurrently, no large change in potential would occur: the action potential arises because the sodium influx and potassium outflux occur consecutively. Such a process, if repeated many times, would ultimately exhaust the internal potassium. Resting conditions are slowly restored by an ionic pump which derives its energy from the general metabolism of the nerve. This pump is usually spoken of as the 'sodium pump' since it is the extrusion of sodium which requires the active expenditure of energy.

When a particular area of membrane is active and possesses a reversed potential, it behaves as an electrical 'sink' drawing positively charged ions from an adjacent area of membrane which acts as a 'source'. The potential across the source is thereby reduced and the membrane is said to be depolarized. One of the consequences of depolarization is a rise in sodium permeability which initiates the sequence of ionic fluxes described above. Conduction is thus a continuous process in unmyelinated nerve, but in myelinated nerve the impulse progresses along the axon in a series of jumps. Myelin is absent at the nodes of Ranvier and only here has the inward sodium current of the impulse been demonstrated[6]. The ionic fluxes that constitute the nerve impulse are thus restricted to these zones, with the result that the impulse is conducted along the axon in a series of jumps, from node to node, at a velocity which exceeds that in unmyelinated nerve fibres.

The action potential or spike lasts approximately 1 msec, but the membrane potential of some neurons may not return to its resting level for up to 6 msec[7]. This phase is known as the period of after-depolarization, or negative after-potential, and is followed by a period during which the membrane potential is actually greater than the resting potential. For example, Coombs, Eccles and Fatt[8] found that the resting potential of a motoneuron in the spinal cord of a cat was approximately -76 mV. Ten milliseconds after a spike the membrane potential measured -81 mV, that

is, the membrane was hyperpolarized by 5 mV. This after-hyperpolarization or positive after-potential, gradually declined until, after about 100 msec, it could no longer be detected. After-potentials, which are almost certainly manifestations of restitutive ionic fluxes[8,9] have the important effect of altering the excitability, and hence the sensitivity of neurons.

Whether the resting membrane is excited by current flow into an adjacent active focus, or artificially by current flow through a stimulating electrode, its potential is reduced. If the current is sufficient to depolarize the membrane by 2 mV, for instance from -70 mV to -68 mV, no spike develops, the membrane potential collapses over a period of several milliseconds to its original level and the depolarization is said to be subthreshold. However, if the current depolarizes the membrane by about 15 mV, a critical level is reached at which an 'all-or-nothing' spike develops. This critical level represents the stage at which the inward sodium current exceeds the outward current of potassium, after which the membrane continues to depolarize and to generate the all-or-nothing action potential. Below the critical level the magnitude of depolarization is graded according to the strength of the stimulus[10]. Since the depolarizing effects of a subthreshold stimulus last for several milliseconds, a second subthreshold stimulus applied within this time will add to the depolarization and, if the critical level is reached, an impulse will be initiated. Hence when the membrane of a nerve fibre is slightly depolarized its excitability is increased. Conversely, where the membrane is hyperpolarized, for instance, from a resting level of -70 mV to -72 mV, then its excitability is depressed, for a stimulus will now have to depolarize the membrane by an extra 2 mV in order to reach the critical firing level. This depression of excitability that accompanies hyperpolarization is known as inhibition.

It follows from these statements that the excitability of a nerve cell should fluctuate with the after-potentials that follow the spike. A stimulus which is just sufficient to reduce the resting potential to the critical firing level should fail to do so during the period of the after-hyperpolarization; and a stimulus which is just subthreshold to the resting membrane should become threshold during the short period of after-depolarization. Wherever it has been looked for, in sympathetic ganglia[11], in neurons of the visual pathway[12] and spinal cord[13–15] of the cat, the correlation between after-potentials and excitability, though not perfect, is good. In addition to these excitability changes the membrane undergoes extreme changes in excitability during the period of the action potential. During the rising phase, a stimulus, no matter how strong, will not add to the spike height or generate another spike, since the membrane is already freely permeable to sodium. As the action potential falls the permeability to sodium is depressed, for a short time, below its level in the resting membrane. During this period the membrane will generate another spike only if the influx of sodium can be made to exceed the efflux of potassium. This requires a larger stimulus while the sodium permeability is depressed; so this phase is known as the period of relative refractoriness. It is followed by the negative after-potential, when this is present.

Up to this point we have assumed that the nerve impulse is initiated by an artificially applied cathodal current, or by local circuits set up by a

nearby active focus, and have avoided the question of junctional transmission. Once it was established[16] that the nerve cell, with its body and fine protoplasmic extensions, the axon and dendrites, formed an independent unit, anatomically distinct from other nerve cells, the question of how signals in one (pre-synaptic) cell were communicated to the next (post-synaptic) was immediately raised. Ramon y Cajal, almost sixty years ago[17], suggested that nerve currents were transmitted from one element to another, across the synaptic junction, by a process of electrical induction. There is, indeed, good experimental evidence[18] that certain synapses in the abdominal nerve cord of the crayfish (*Astacus fluviatilis*) operate by electrical transmission, but no good evidence that vertebrate synapses operate in this way[19].

The electrical transmission theory was first seriously questioned as a result of the experiments of Dale, Feldberg, and Vogt[20]. These workers found that when motor nerve fibres to a skeletal muscle were stimulated, a minute amount of a chemical substance, acetylcholine, could be detected in the fluid perfusing the muscle. Direct application of this substance to the neuromuscular junction[21,22], led to the discharge of impulses in the muscle membrane. These experiments all strongly suggest that the muscle membrane was depolarized through the action of acetylcholine liberated by the axon terminals. Recent work[23,24] has lent support to this view and given some insight into the mechanisms underlying the transmission process. Fatt and Katz[25], recording from the muscle membrane at the neuromuscular junction, observed irregular fluctuations in the membrane potential, of the order of 0·5 mV. Each of these was thought to be generated by the depolarizing action of a minute quantity of acetylcholine. This hypothesis has received some interesting correlative support from recent observations on the fine structure of axon terminals at the neuromuscular junction[26]. The terminals (*Figure 6.1*) contain large numbers of small (synaptic) vesicles, and it seems reasonable to suppose, in the absence of direct evidence, that the transmitter substance is contained within them*. The spontaneous extrusion and bursting of one of these vesicles would then generate a miniature spike in the junctional muscle membrane. An impulse in the nerve fibre might lead to the synchronous release of large numbers of these vesicles and, consequently, to the generation of a corresponding number of miniature spikes. These summate[25,28] and by local circuit action may initiate an impulse in the muscle membrane. Within a fraction of a second after its liberation acetylcholine is inactivated by the enzyme cholinesterase which is concentrated[29] in the muscle membrane at the neuromuscular junction.

It is a far cry from the neuromuscular junction to the neuroneuronal synaptic junction of the central nervous system, but there are many features of structure and function which are common to both. Whenever synaptic knobs of the vertebrate central nervous system have been examined under the electron microscope[30,31] synaptic vesicles have been recognized. Not all pre-synaptic terminals end in knobs, however, nor is it certain that vesicles are present at all synaptic junctions. Nevertheless, the evidence is

* Ladman[27] has described synaptic vesicles in cell processes on both sides of the junction between photoreceptors (rods) and bipolar cells in the vat retina. If the vesicles contain transmitter substance, these findings raise the question of whether or not some synapses operate in two directions.

good[9], though not unassailable, that chemical transmission forms the basis of interneuronal communication in vertebrates. The surface of the cell body (soma) and basal dendrites of many neurons in the central nervous

Figure 6.1. Electron micrograph from a myoneural junction of the frog sartorius muscle in longitudinal section through the muscle fibre[26]
Synaptic vesicles (sv) and mitochondria (m) are seen in the axon terminal (N) which is closely applied to the muscle fibre (M). On the opposite surface of the nerve terminal may be seen a Schwann cell (sc) and connective tissue fibrils (ct)

system are encrusted with synaptic knobs, which may be derived from many nerve fibres converging on the cell. In motoneurons of the spinal cord, a volley of impulses in pre-synaptic fibres is thought to liberate an excitatory transmitter substance which depolarizes the sub-synaptic membrane[7]. If this is insufficient to generate an impulse, a second volley, if appropriately

timed, will bring about further depolarization (temporal summation), to which may also be added any excitatory activity initiated at adjacent synapses (spatial summation). Local circuits are set up between the excited sub-synaptic membrane and the adjacent resting membrane which is thereby depolarized. A spike is generated in the soma when its membrane is depolarized by 20 to 37 mV. At the axon hillock, the junctional zone between axon and cell body, the critical level of depolarization is 5 to 18 mV[32]. It is at this junction that the propagating spike is first generated in the motoneuron[32] and therefore in other nerve cells[33,34]. The spread of depolarization from the active synapses to the axon hillock is probably aided by the generation of partial spikes in the dendrite and soma membrane[35,36]. The intense current flow at the axon hillock set up by the propagating impulse, is usually sufficient to further depolarize part of the cell body which now also generates a large spike.

When a cell is discharging impulses, further release of the excitatory transmitter substance will increase its firing rate; where a cell is not discharging the effect of the excitatory substance will be either to throw it into activity or, by causing subthreshold depolarization, to increase its excitability. Not all transmitter substances depolarize the membrane, however. Where they hyperpolarize, depressed excitatory is manifest as a failure to discharge to a previously threshold excitability stimulus, or, where the cell is active, as a reduction in the rate of firing. Evidence has recently appeared[37,38] which demonstrates that inhibitory effects may also be brought about by interfering with the release of excitatory transmitter substances from axon terminals. This process is known as pre-synaptic inhibition.

A particularly important form of inhibitory activity, and one which probably operates at all levels of the central nervous system, was discovered by Renshaw[39] in the spinal cord of decerebrate, lightly anaesthetized cats. The object of his experiments was to investigate the functional significance of the fibres (recurrent collaterals) which arise from many primary motor nerve axons a short distance from the axon hillock[40] and sweep backwards to terminate among other neurons in the ventral horn of the spinal cord. The afferent dorsal roots of a spinal nerve were stimulated, and reflex (orthodromic) activity thereby elicited in a number of nerve fibres passing to a muscle. Another group of motor nerves to the same muscle was stimulated so that a volley of impulses passed backwards (antidromically) to the spinal cord. It was found that the antidromic volley reduced the size of the reflexly elicited motor discharge, and Renshaw concluded that these inhibitory effects were mediated by the recurrent collateral branches of the antidromically activated cells. Since the collateral branches are likely to be active whenever the parent axon conducts impulses, recurrent inhibition will also occur whenever the motoneuron is active. Renshaw[41] later showed that, associated with the arrival of the antidromic volley in the spinal cord, there was a burst of action potentials which could be recorded with a microelectrode, and which he thought was due to the firing of interneurons. He postulated that recurrent collaterals terminated on these interneurons (now known as Renshaw cells) and that the inhibitory effects on motoneurons which he had previously described were directly attributable to activity in these cells. There is now good evidence to suppose that this is indeed the

case, for the inhibitory effects can be depressed by depressing the activity of the Renshaw cells[42]. The discharge of a particular Renshaw cell can be influenced by activity in several motoneurons[39,42] so it seems probable, though not certain, that the inhibitory action of such a cell is also exerted on the motoneuron that drives it.

Recurrent inhibition, like the positive after-potential, is an impulse-dependent process which tends to depress the excitability of a cell after it has been conducting. Presumably the faster a neuron with collaterals discharges, the greater will be the recurrent inhibition. Where the Renshaw cell feeds back on to the neuron which drives it there will be algebraic summation of excitation and inhibition. If the excitatory stimulus were well above threshold, the neuron would continue to discharge although, checked by recurrent inhibition, at a slower rate. If this highly activated neuron lay in a group of nerve cells which were receiving near-threshold stimulation, those neurons close to it would be fully exposed to the intense Renshaw cell discharge. The excitability of these cells would then be depressed and they would cease to discharge. In such a group of neurons, highly activated single cells would be surrounded by a zone of relative quiescence. Mutual inhibitory interaction between adjacent cells would always tend to favour the more intensely excited cell on which the 'depolarizing pressure'[43] was greatest, and choke off activity in those cells in which the depolarizing pressure was near threshold. As we shall see (p. 262 *et seq.*), mutual inhibition is, quite certainly, of fundamental importance in the functional organization of the central nervous system in general.

THE CODING OF SENSORY INFORMATION

A code is a set of signs or symbols into which messages may be transformed. The transformation must be reversible so that given a particular set of symbols together with the key to the code, the original information may be reconstructed. A good example is the Morse code where a verbal message is transformed into a sequence of dots and dashes. These dots and dashes constitute the signs of the code. The signs used in the central nervous system are, so far as we know, the all-or-nothing impulses. Whether graded potentials select and code information[44] other than through their effects on the nerve impulse (p. 245) is not known, but is a question on which we should keep an open mind. For conduction over long distances, however, the action potential appears to be the significant sign.

If we wish to know how the central nervous system deals with the information it receives and stores, we need to understand the code it uses to transmit sensory messages. One of the ways of obtaining this information is to record the patterns of impulses elicited by a variety of sensory stimuli and then to see whether any rules can be formulated which accurately describe the relationship between the various sensory inputs and the neuronal responses which they evoke. Stimulus properties can be divided into those of form (*e.g.* size, shape), intensity (*e.g.* brightness, weight) and sensory quality (*e.g.* photic, acoustic) and what is known of the coding of these three parameters will be discussed in the following pages. Since techniques for studying the electrical activity of single units (nerve cell bodies and their

processes) in the central nervous system have only recently become available, it is not surprising that our knowledge of the structure of the sensory code is still rudimentary.

Intensity

Biological coding mechanisms incorporate a process whereby energy is transformed from one state into another. Any such process is known as transduction, and a device which performs this operation is known as a transducer. A microphone is a transducer since it converts mechanical energy (sound waves) into electrical energy. The sense organs are transducers since they initiate a discharge of impulses when activated by an appropriate stimulus. The stimulus, which may take the form of radiant, mechanical or chemical energy, must usually be quite specific if it is to activate a given receptor—a muscle stretch receptor is not activated by light and a photoreceptor is not activated by tension. In contrast, there are two aspects of the transduction process which may not be receptor specific. In some way the stimulus-induced change in the state of the receptor must be transformed into a change of electrical potential across the membrane of the nerve cell and, secondly, this potential change must be related in some way to the propagation of action potentials in the sensory fibre. At many synapses in the vertebrate central nervous system, and at the vertebrate neuromuscular junction, a transmitter substance, liberated at the axon terminals depolarizes the post-junctional membrane and it seems reasonable to suppose that a similar mechanism may operate at certain neuroreceptor junctions. Evidence of such neurohumoral transmission is equivocal for many neuroreceptor junctions[45], being best in the case of the photoreceptor nerve cell junction in the lateral eye of the horseshoe crab, *Limulus*[46]. Because of its theoretical importance the work on *Limulus* will be described in some detail.

The compound eye of the horseshoe crab possesses some 800 ommatidia. Each ommatidium contains a photoreceptor which is thought[47] to lie in close relationship to the dendrites of a cell, the eccentric cell, which sends its axon into the optic nerve. When light impinges on the receptor a photochemical reaction[48] takes place leading to a discharge of impulses along the axon of the eccentric cell[49]. Fuortes[46] working with the excised eye, isolated, with a micropipette electrode, a number of units which he tentatively identified as eccentric cells. The microelectrode, which was a very much finer version of that used by Hodgkin and Huxley[1] in the squid axon, was thrust into the eccentric cell to record intracellular potentials. Where a cell, penetrated in this way, exhibits stable resting and action potentials it is unlikely that the electrode can have done it much damage. This view is strengthened by the fact that spike activity elicited by light is virtually the same, whether one records from the impaled cell[50] or from its intact axon in the optic nerve[51]. The response of an impaled cell to prolonged illumination was found to consist of a sustained depolarization (the generator potential) with superimposed spikes. The depolarization evidently spread over the dendrites to some trigger area, proximal to the cell body, and there elicited a rhythmic discharge of propagating action potentials. So long as

the generator potential persisted, the repetitive firing continued. This relationship between generator potential and spike activity has been observed in other sensory neurons[52–55] and is thus not unique to the eccentric cell of *Limulus*. Changes in permeability of the eccentric cell membrane accompany and, by allowing certain ions to cross the membrane, probably cause the generator potential. Since these permeability changes could not be induced by an electric current passed through the recording electrode, it seems unlikely that they can be caused by current flow from an active photoreceptor. Fuortes[46], therefore, postulates that transmission between photoreceptor and eccentric cell is chemical and not electrical.

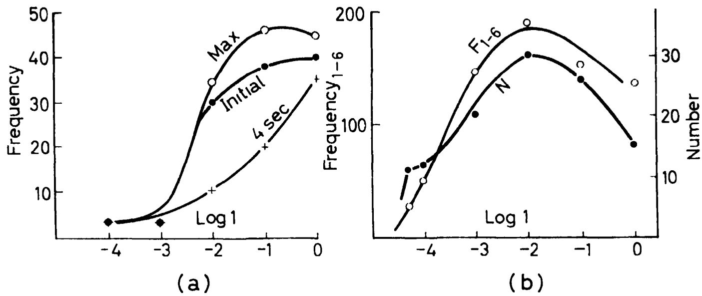


Figure 6.2. The relationship between logarithm of intensity of retinal illumination (abscissae) and discharge (ordinates) in single optic nerve fibres of the frog's eye[57] (*a*) The initial and maximum frequencies of the initial burst response of a unit which was excited by light, together with the frequency attained after 4 sec of steady illumination. (*b*) Graph relating frequency of the first six impulses (left-hand ordinate) and total number of impulses (right-hand ordinate) in the burst, to light intensity, in a unit which gave a response only when the light was extinguished

The frequency of action potentials in a single fibre of the optic nerve is linearly related to the logarithm of the intensity of light falling on the corresponding ommatidium[51], so that a tenfold increase in the intensity of illumination causes a constant increase in the number of spikes generated. Fuortes[46,50] found that impulse frequency was a linear function of generator potential amplitude, but that the latter was a linear function of the logarithm of light intensity. Thus the logarithmic transformation takes place at some stage prior to the appearance of the generator potential. A similar relationship between stimulus intensity and spike frequency has been demonstrated by Mathews[56] in the sensory fibre from the frog stretch receptor. However, while a logarithmic transformation probably characterizes most sensory transducer processes, the function is not always linear (*Figure 6.2*) so that it is not at present possible to formulate a general equation that adequately describes, for all sense receptors, the relationship between stimulus intensity and impulse activity in the sensory nerves. Nevertheless, for a given receptor and its nerve fibre, and over a specific range of stimulus intensities, it should be possible to make a preliminary analysis of the intensity code.

By constructing an impulse frequency–stimulus intensity curve and using this as the key, we should be able to read off stimulus intensity for any

spike frequency that falls within the limits of the curve, and so decode the message. However, if we followed this plan of action we could, for several reasons, easily be misled. One nerve fibre may innervate several receptors in the skin; for example, a single fibre may send branches to between 40 and 120 hair follicles[58] and where this happens, the information transmitted by an increase of spike frequency would be ambiguous. It could, in this particular example, signify either an increase in magnitude of the weight bending a single hair, or an increase in the number of hairs being bent, or both. This sort of ambiguity (which may exist in sensation) immediately limits the value of the impulse frequency–stimulus intensity curve, and implies that further information is required to decide what is happening at the sensory surface. Furthermore, a single hair follicle receives branches from between two and six sensory fibres[58], so that a weight applied to one hair may evoke spike activity in, and hence lead to the distribution of information among, this number of neurons. Once the first synaptic junction between primary and secondary afferent neuron has been reached the information may be even more widely dispersed. A single axon may break up into many terminal branches which make contact with several post-synaptic nerve cells, and each of these may receive terminals from many pre-synaptic axons. In the primate retina, for example, a single bipolar cell (primary afferent neuron) may synapse with between three and four ganglion cells (second-order neurons) and each of these may synapse with anything from one to several hundred bipolars[59]; in the ventral cochlear nucleus (the first synaptic junction in the auditory pathway) of the cat, it is estimated[60] that each primary afferent fibre influences 400 secondaries and each secondary neuron receives inputs from 400 primaries.

All these facts strongly suggest that, as a general rule, information about the intensity of even highly localized stimuli can be recovered only by cross reference to activity in a number of sensory channels and by analysis of impulse frequency in each of them. However, any preconceived notion of a simple relationship between impulse frequency and stimulus intensity, such as the one we started with, will have to be abandoned, because of the risk that a number of spikes have been interpolated into the fibre through the activity of previously quiescent receptors to which it may be connected. Furthermore, mutual inhibitory interaction between sensory cells would lead to a reduction in the rate of impulse activity in a given fibre, and further complicate the relationship.

Clearly, to evaluate the information transmitted by a single fibre, and in particular, to decide whether a change of impulse frequency signifies a change in the intensity of the stimulus, it is necessary, once again, to know what is going on in channels carrying information from other receptors. The extraction of information about stimulus intensity thus requires elaborate computation. This could be circumvented to some extent, if, instead of absolute intensity at a focus on the sensory surface, it were sufficient to know the intensity at that focus compared to the intensity elsewhere. After all, this, and not the absolute intensity, is what matters to the animal.

When the stimulus is large and activates many receptors, interaction between conducting neurons may be considerable; this will be discussed in another section (p. 262 *et seq.*).

Implicit in all the statements that have been made so far is the assumption that nerve fibres which connect with sense organs are thrown into activity only when a suprathreshold stimulus impinges on the receptor. This assumption, however, is probably false and is refuted by a number of experiments. Hoagland[61,62] and later Sand[63] recorded from sensory fibres connected to lateral line sense organs and found that impulses could be recorded from these fibres even when the stimulus which normally provokes the receptors was almost certainly excluded. Tasaki[64] found fibres in the auditory nerve of the guinea-pig, which conducted impulses in the absence of acoustic stimulation. Spontaneous activity has also been demonstrated in sensory fibres from statocyst receptors in the lobster[65]. It is, of course, very difficult to be quite sure that a receptor is not being activated by stray stimuli, but it does seem possible to be sure of this in experiments on vision. It is possible, with care, to exclude all light from the preparation and, in the cat retina, impulse activity has been recorded from ganglion cells[66], and from units which were probably bipolar cells[67], in complete darkness.

There are several possible causes of this unprovoked activity. It might arise from spontaneous changes in the state of the receptors (*e.g.* spontaneous breakdown of pigment in photoreceptors) or be generated by random ionic activity in the dendritic membrane of the sensory neuron[68]. Where neuro-humoral transmission occurs, it might be caused by the spontaneous release of transmitter substance from receptor endings when quanta of the substance, as a result of thermal agitation, collide with and occassionally pass through pores in the pre-synaptic membrane (*cf.* p. 246). Besides, or in addition, spontaneous oscillations of the transmembrane resting potential, which were probably responsible for the repetitive discharges of decalcified *Sepia* nerve, described by Arvanitaki[69], may be a contributory factor.

The fact that spike activity occurs in an afferent pathway in the absence of sensory stimulation introduces yet another variable to the already complicated relationship between stimulus intensity and spike frequency and has a number of important consequences. It confers, as it were, another dimension for registering sensory change. Thus, single fibres in the vestibular nerve of the cat[70] show spontaneous activity when the head is kept in the horizontal plane. When the head is rotated to the right, the rate of discharge of some fibres increases and when the head is rotated to the left, the rate of discharge of these fibres decreases. This system of coding information about spatial orientation could not, of course, be used in the absence of spontaneous impulse activity.

A problem of a different kind, imposed by the presence of spontaneous spike activity, derives from the fact that a given train of impulses may be elicited by an external stimulus, or occur independently of external stimulation. How are we (or for that matter, how is the brain) to distinguish between these possibilities, for if we are unable to do so, we may infer the presence of a stimulus when none is there or, conversely, fail to recognize one that is? The problem is not a uniquely biological one, for the same sort of problem arises in any communication system. When we listen to someone speaking on the telephone the voice may almost be submerged by interfering crackling sounds; or the continuity of a picture on a television screen may be disrupted by irregular bands of black and white spots. In

general, the word 'signal' refers to the message that is to be transmitted and 'noise' refers to any unwanted disturbance, introduced at any stage in the communication system, that interferes with the reception of the message. In the telephone example, the voice is the signal and the crackling sound is noise. If the crackles are weak and voice loud, there is no difficulty in hearing what is said. That is, the intensity (energy) of the signal greatly exceeds that of the noise, the signal noise ratio is high, and the message is easily extracted. When there is a great deal of crackling on the line and the voice is barely audible, the signal noise ratio is low, and the message is difficult to extract. As a result we may not hear, or may misinterpret what is said. Where noise is present in the absence of a signal, and spontaneous neural activity is a case in point, then the magnitude of the noise must set a limit to the sensitivity of the system[71]. Thus, if a single quantum of light activates a rod in the retina and so generates an impulse in a ganglion cell, there is nothing to distinguish this spike from a spontaneous discharge, so that the information carried by the spike is ambiguous. It seems then, that our judgement on whether or not a near-threshold stimulus has been presented is based on guess-work in which we have to decide, on the basis of probabilities, whether or not a particular train of impulses could be accounted for as a random fluctuation in the level of neural noise. When the stimulus is so intense as to greatly exceed the intrinsic noise in the sensory pathway, the probability of making an error because of this noise approaches zero.

To sum up, impulse frequency signals intensity, but it may be the intensity of some aspect of the stimulus other than that used by the physicist, *e.g.* 'off' units in the retina (p. 263) signal 'darkness'. The relationship between impulse frequency and intensity is not simple. It is complicated by the fact that a single receptor is rarely connected to a single fibre, or a single fibre to only one receptor, and this divergence and convergence of sensory channels also occurs within the central nervous system. The relationship is further complicated by mutual inhibitory interaction between the neurons that conduct signals to the brain, and by the fact that many, probably all, of these cells show spontaneous impulse activity.

Physical Properties

Objects in the physical world possess different physical properties. Some generate pressure waves, others emit electromagnetic radiations and all have mass. These properties must fall within a certain range of values if we are to be able to detect them at all. We experience as light those electromagnetic radiations which are in the wave-length range of 400 to 700 mμ, but we do not appear to have any direct experience of waves that are much shorter (x-rays) or much longer (radio-waves). Similarly, we experience as sound pressure waves with a frequency varying from a few cycles per second to about 15,000 c/s, but frequencies much above this are inaudible. Within this range of detectability the physical properties of an object activate receptors and thus operate as sensory stimuli giving rise, under certain conditions, to sensory experiences in consciousness. In the discussion which follows we shall not be concerned with the problem, if there is one, of the relationship between physical property and sensory quality (for example, how to relate the subjective experiences of redness to electromagnetic

radiations in the wave-length range of 647 to 723 mμ), but with the question of how the physical properties of an object are coded for transmission within the nervous system.

If there were only one sort of receptor* at the sensory surfaces of the body, which responded in a uniform way to any form of energy, we could discriminate different stimuli only on the basis of differences of intensity, size or shape. We should not be able to discriminate on the basis of different physical properties since we should, presumably, have experience of only one sensory quality. In fact, there are different receptors and these are selectively sensitive to certain forms of energy (the receptors associated with the sensation of pain are an exception to this rule). It seems reasonable, therefore, to suppose that this specificity has something to do with the coding of those physical properties of an object which are physiologically detectable. There is, indeed, no reason to doubt the validity of this supposition, but there are some derivative problems, the solutions to which are not intuitively obvious. Thus, while a given receptor is selectively activated by one form of energy, its selectivity may not be absolute. Hensel and Zotterman[73], for example, recorded the discharge of single fibres in the lingual nerve of the cat and found some units which could be activated by mechanical *or* thermal stimuli applied to the tongue. Thus there may be some ambiguity in the message carried by such a fibre with the consequent loss of information.

In the event, it seems that the coding of major energy forms which correspond respectively to auditory, photic, olfactory, gustatory and somaesthetic sensory qualities, is a relatively simple affair. Sensory quality appears to be assigned according to the anatomical channel along which afferent signals are conducted[74]. Thus signals in the optic pathways are normally elicited by changes in the intensity of light falling on the retina, and there is good reason to suppose that any message passing along these pathways, however initiated, gives rise to a visual sensation. Pressure on the eyeballs produces a sensation of light and so does an electric current passed through the eye. Brindley[75] has produced evidence which suggests that the electric current activates retinal photoreceptors or the neurons on which they synapse.

More direct and dramatic evidence in support of the hypothesis that different forms of energy are coded by an arrangement which is channel-dependent, comes from the work of Penfield and his collaborators[76,77] on human subjects whose brains have been exposed for certain neurosurgical procedures. It is not always necessary to administer a general anaesthetic to the patient at these operations since, after the dura mater has been incised and reflected, further operative manipulation of the brain is quite painless. Once the brain has been exposed the surface of the cerebral cortex may be stimulated by current passed through ball-tipped platinum wires, and since the patient is conscious, he is able to describe any sensations that may be

* The term 'sense organ' refers to a specialized mass of tissue, supplied by a sensory nerve, and having low threshold to some particular form of energy. Muscle spindles, Pacinian corpuscles, rods and cones are examples of sense organs. It has become increasingly clear over the past few years[72], however, that areas of skin with bare nerve endings, and apparently devoid of sense organs, discriminate a variety of stimuli, and it is difficult to avoid the conclusion that bare nerve terminals may act as transducers. The term 'sense receptor' will therefore be used to refer to any biological transducer, whereas the term 'sense organ' will be used to refer to those receptors which have a specialized structure and are adapted to respond to a particular type of stimulus.

elicited in this way. When the stimulating electrodes were placed on or beneath the visual cortex[76], which receives extensive input from the optic pathway, and a current passed, the patients described visual images of a rather simple kind. These included stars, diamonds, coloured balls, flashing lights and moving wheels. Following stimulation of the auditory cortex, patients heard sounds which they described as ringing, buzzing, drumming, booming, and sometimes compared the sounds to the noise of crickets, a bell or a whistle. Stimulation of the olfactory bulb, a nucleus in the olfactory pathway, was regularly associated with sensations of smell which were almost invariably disagreeable[77]. Sensations of taste could also be elicited following stimulation of the appropriate cortical area, but none of the patients that had been examined described the taste as pleasant; it was always bitter, or disagreeable or undefinable. Stimulation of the somatic sensory cortex resulted in sensations described by the majority of patients as tingling, numbness, or a feeling of electricity.

It will be noticed that the sensations elicited by cortical stimulation were, in general, of a relatively crude and undifferentiated kind. This is not, perhaps, surprising since the stimulus is also crude. It seems reasonable to suppose that where electrically excitable neurons fall within the field of influence of a suprathreshold electric current they will be discharged, and that the delicately balanced patterns of interaction that are brought into play by relatively simple physiological stimulus will be grossly disorganized. However, this cannot be the whole story for Penfield and Jasper[77] describe a few cases in which electrical stimulation of the cortex, usually outside the primary sensory areas, was followed by vivid perceptual experiences of a most complex kind—a boy heard his mother talking on the telephone and saw robbers coming towards him with guns, another saw men fighting and a woman heard an orchestra play a well known tune. Whatever the significance of these observations, the work of Penfield and his colleagues lends much experimental support to the view that when a signal is channelled into a particular sensory pathway in the nervous system its general quality or modality (*i.e.* somaesthetic, olfactory, and so on) is thereby established.*

Within a sensory pathway, the code used to transmit information about the nature of a stimulus appears to be quite complicated and to depend much less on the anatomical position of the pathway. Thus single fibres in the auditory nerve of the guinea-pig[64] respond to a narrow band of frequencies when the stimulus is at near-threshold intensity. If we could somehow label each fibre and assign its corresponding response characteristics at threshold then, when a given fibre was active, we could easily deduce the approximate frequency of the stimulus. The nature of the stimulus would thus be assigned by channel-identification. As the intensity of the stimulus is increased above threshold, however, the fibres come to be activated by a wide range of frequencies (*e.g.* 1 to 8 kc/s), although each fibre shows a very sharp cut-off frequency above which it does not respond. Activity in one of these fibres would then indicate that a stimulus had been applied, but would provide little information about its frequency or pitch. To extract this information we would need to know what was going on in

* Recent work[145] has shown that some cells in the visual cortex respond to non-visual sensory stimuli (see *Figure 6.8*) and these results bring an element of uncertainty into the general validity of the above view.

other fibres of the auditory nerve. In fact, a pure tone, with a frequency greater than approximately 500 c/s, and if sufficiently intense, will set up a travelling wave along a variable length of the basilar membrane[78,79]. The amplitude of this wave is maximal at a restricted region, and here we may expect that the sensory nerve fibre will fire at a higher rate than fibres from adjacent regions. The basal turn of the organ of Corti is essential for hearing tones of high frequency, and injury to the apical portion of the basilar membrane often causes a loss of sensitivity to notes of low frequency[80]. Thus, to extract information about the frequency of a particular auditory stimulus from the pattern of impulse activity generated by it, we should need to know this tonal organization of the basilar membrane, and the anatomical locus on the membrane from which each fibre in the auditory nerve arises. We could then deduce, from the distribution of active fibres within the nerve, the *range* of frequencies within which the stimulus fell. This is as far as channel-identification would take us. To obtain more precise information about the stimulus we should have to compare the firing frequency of the active fibres to decide which had the highest rate of discharge. This information appears to be necessary for fine frequency discrimination, but it is not sufficient, and it is certain that a number of other factors, such as the effects of neural interaction[81] and, possibly, the temporal distribution of spike discharges among the array of active fibres, would have to be taken into account.

Another departure from channel-dependent coding is found in the pathways of taste. Pfaffman[82] recorded the discharge of single fibres in the chorda tympani of rats after flooding the tongue successively with solutions of sodium chloride, potassium chloride, hydrochloric acid, sucrose and quinine hydrochloride. As index of response, he counted the number of impulses during the first second following the application of the stimulus. He found, as we might expect, that an increase in stimulus intensity (concentration) was associated with an increase in both frequency of impulses per fibre and in the total number of fibres active. Further, in the nine single fibres that he analysed in detail, eight showed a response to at least two of the test solutions, and five a response to at least four of the test solutions. One fibre, for example, gave a burst of 25 impulses in the first second after a solution of hydrochloric acid had been applied, and 18 impulses after a solution of potassium chloride had been applied. We could learn very little about the nature of the stimulus from a unit such as this if we knew only that it was firing at a frequency above its spontaneous rate of discharge. Nor could we learn very much more if we knew its precise rate of firing, since, presumably, 25 impulses/sec could signify hydrochloric acid solution, or a more concentrated solution of potassium chloride than that applied in the above experiment. *Figure 6.3* illustrates this dilemma. The unit B discharged approximately 15 spikes in the first second after application of a molar solution of sodium chloride and the same number of spikes after application of an approximately 0·1M solution of sucrose. Information about the intensity and nature of the stimulus is thus confused and cannot be separated on the evidence provided by this fibre alone. However, if we know what was going on in other units, such as A, we should be more favourably placed to make this distinction. Both units are stimulated by

sodium chloride and by sucrose, but A responds more briskly to a given concentration of sodium chloride and B responds more briskly to a given concentration of sucrose. Thus the salt stimulus is indicated when A is more active than B, and the sweet stimulus when B is more active than A. Intensity would be given by the actual rate of discharge of each fibre. It seems that sub-qualities of taste are assigned according to: (*a*) which fibres are active, and (*b*) the distribution of impulse activity within this array of discharging fibres.

An additional method of coding energy form is found in the visual system. Liberman[83] described a number of ganglion cells in the frog retina which

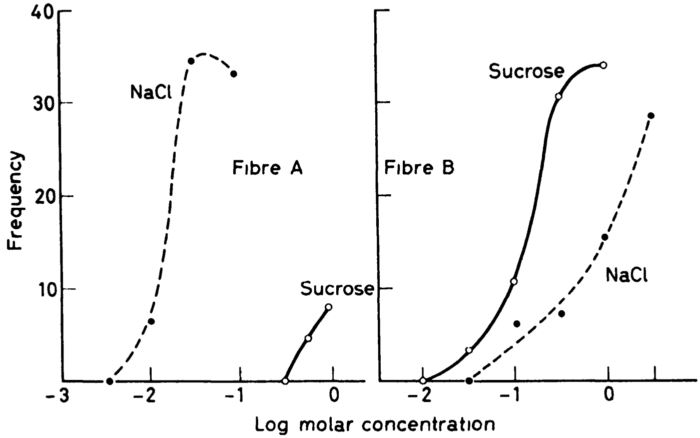


Figure 6.3. Curves relating impulse frequency to concentration of two test solutions, sodium chloride and sucrose, for two fibres of the chorda tympani in a rat[82]. Each fibre gave a response to both solutions, but fibre A was relatively more sensitive to sodium chloride and B was relatively more sensitive to sucrose.

gave a different pattern of response when stimulated by light of different wave-lengths. One such cell showed spike activity throughout the period of illumination by blue light, but only at the onset of illumination by red light. Information about stimulus intensity was transmitted independently of wave-length, for when the intensity of each stimulus was increased, the number of spikes generated increased, but the temporal pattern of discharge remained the same. De Valois, Smith, Kitai and Karoly[84] have shown similar evidence for wave-length coding in the visual system of the monkey. Ganglion cells in the goldfish retina also respond differently according to the wave-length of light rays[85]. The unit illustrated in *Figure 6.4* was excited by light of short wave-length and inhibited by light of long wave-length, and the transition from excitation to inhibition was quite abrupt. Wolbarsht, Wagner and MacNichol[86] have suggested that these two mutually antagonistic processes arise from independent peripheral pathways, each connected to photoreceptors with different spectral sensitivities. Thus, evidence is emerging[86,87] that the sort of cross referring which is required to sort out the sensory quality of a stimulus, and to generate different patterns of spikes in a given ganglion cell, may be achieved by inhibitory connections between the different receptor assemblies.

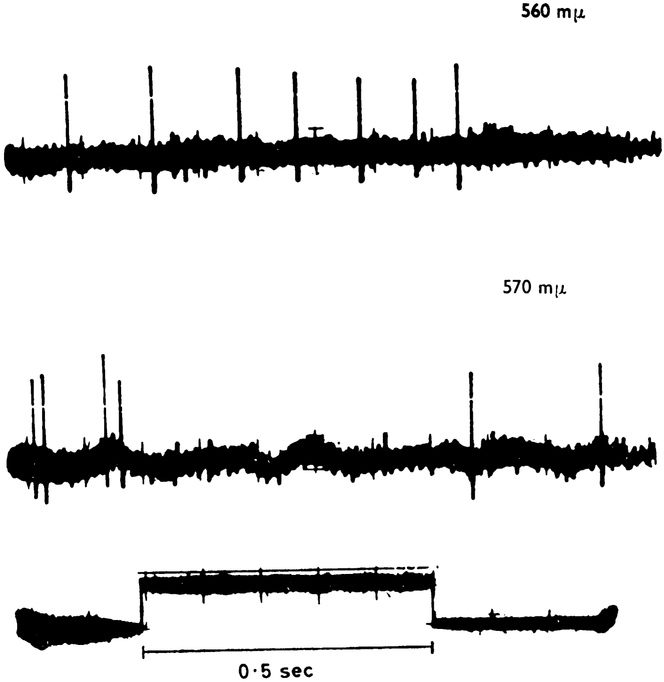


Figure 6.4. Response of a single ganglion cell in the goldfish (*Carassius auratus*) retina to small changes in wave-length of light[85]. Duration of stimulus indicated by in step signal record.

Form

The eyes of human beings, though not of all mammals, can be moved independently of the head, so that when the head is fixed the eyes can be rotated in the orbit and thus used to scan the fields of vision. These movements are obvious. What are not obvious are the small oscillatory and step-like (saccadic) movements that occur when we look at an object as steadily as possible[88–90] and which impose a continuous relative movement between retina and retinal image. The fact that these movements occur suggests that they may be of some importance for visual perception. In the past few years a number of investigations have been designed to explore this possibility by using a device which maintains the image of an object stationary on the retina. This condition can be established by the use of a contact lens applied to the surface of the eye. The technique used by Ditchburn and Ginsborg[91] was as follows. A small mirror was mounted on the contact lens. The image of a test object (a vertical bar between two regions of differing brightness) was focused on this mirror and was there reflected on to a screen where it could be seen by the eye. Since the contact lens moved with the eye, so did the mirror and so also did the image reflected

on the screen. In this way the image of the vertical line was stabilized on the retina. Within a short time after it had been presented, the subject reported that the stabilized retinal image of the vertical bar had disappeared. After a short time it reappeared, and this sequence of disappearance followed by spontaneous regeneration was continued at irregular intervals. In the light of more recent work[92,93], the possibility that the re-appearance of the image was an artefact, due to the contact lens slipping over the surface of the eye, has not been excluded. Evidence in favour of this view comes from the work of Iarbus[93] who used a small contact lens firmly applied to the cornea by suction. The object to be viewed was mounted on the contact lens. Iarbus found that the retinal image, stabilized in this way, faded rapidly but could be made to reappear by relative movement between eye and contact lens. Campbell and Robson[94] have since confirmed, with a technique which avoided the use of contact lenses, that a stabilized retinal image disappears and never reappears spontaneously.

The importance for visual perception of relative movement between stimulus and receptive surfaces makes one wonder whether the visual system is unique in this respect. Quite certainly it is not. When the hand comes in contact with an object, we often run our fingers over its surface to learn something about its texture, size and shape. If the exploring fingers come to rest, even for a short time, the amount of information which we receive about the object is greatly reduced. An odour may be detected easily when it first reaches us, but we are soon unable to smell it unless we sniff and so draw currents of air over the olfactory epithelium. The same temporary adaptation can be shown in the ear. Caussé and Chavasse[95] delivered a pure tone to a subject after which they measured the auditory threshold for this and other tones. They found that the threshold for the test tone was slightly raised for a short time, other tones being unaffected.

We may conclude that, to an extent that varies from one modality to another, relative movement between stimulus and sensory surface is an important and necessary condition for perception. This fact suggests that each sensory system possesses a mechanism, operative when stimulus conditions at the sensory surface are invariant, which attenuates the sensory inflow to a constant stimulus. The existence of such a mechanism is hardly surprising from a teleological point of view. For both predator and prey, moving objects have much greater survival interest than stationary objects.

The blunting of sensory experiences when the relationship between stimulus and receptive surface is held constant, is probably an expression, at the level of perception, of the differentiating properties of sense receptors. These properties were first discovered by Adrian and Zotterman[96] in the cat and have since been shown to characterize the behavior of sense receptors in many other animals. Adrian and Zotterman found that when a weight was lowered on to a cat's toe pad, a discharge in the nerve from the pad lasted from 0·1 to 0·2 sec and then subsided, even though the weight remained on the pad. This decline of impulse activity in the continued presence of the stimulus, is known as adaptation. The time course of the adapting process varies from one receptor to another. In contrast to the rapid decline in rate of firing of the afferent nerve leading from the toe pad receptor, the nerve supplying a stretch receptor in frog muscle may continue to discharge to a

maintained stretch for as long as 10 minutes, although at a lower rate than the high frequency, rapidly declining, burst seen at the beginning of the stretch[97].

If we assume that impulse activity in retinal ganglion cells is gradually choked off when a light of constant intensity falls on its receptive field* then, together with what we know about eye movements and the properties of stabilized retinal images, we can predict how information about a vertical line may be transmitted in the optic pathways. If the image of a fine vertical bar of light is focused on the retina, it will excite an array of photoreceptors and so initiate a change in the rate of impulse activity in those ganglion cells with which the receptors are connected. An involuntary movement of the eye will then bring another column of receptors, several cone diameters away from the first position, beneath the image of the bar, and this process will continue at a rate determined by the frequency of the involuntary movements. Thus a vertical line, even if its width is close to the diameter of a single rod or cone, may be expected to generate activity in more than one column of photoreceptors and intermittent ('on–off') activity in the associated ganglion cells. This hypothesis is supported by the fact that the stabilized image of a vertical line may be clearly seen whenever there is sufficient relative movement between eye and retinal image to displace the latter more than one cone diameter across the retina[100].

Although it is clear, from the experimental evidence outlined above, that eye movements are necessary for detailed vision, common sense suggests that they will blur the image seen. If this supposition is correct we can expect the stabilized retinal image to be sharper in the first few moments after it is presented than when the image is seen with normal eye movements intact. In the former condition the image is stabilized across an array of receptors, movement is eliminated, and any blurring due to such movement also eliminated. In the event, Riggs, Ratliff, Cornsweet and Cornsweet[92] found that when a stabilized retinal image was first presented, fine lines were seen with normal or better than normal acuity. These observations support the earlier ones of Ratliff[101] who found that for involuntary movements within a certain range of amplitude, monocular visual acuity decreased as the amplitude of the movement increased.

When the image of an object is stabilized on the retina, the receptor assembly may be expected to adapt, and evoked impulse activity in the optic nerve fibres to be cut off. This hypothesis, which could account for the fade-out of stabilized retinal images, receives some direct support from the work of Hartline[102] on the frog retina. He found that when a dark line was focused on the receptive field of ganglion cells, no impulse activity occurred so long as the line remained stationary. When the line was moved from one set of receptor elements to another, a brisk burst of impulses was generated. Kuffler, FitzHugh and Barlow[103] also described rapidly adapting ganglion cells in the cat's retina, whose spontaneous rate of discharge was only transiently changed by light shone on to their receptive fields. However, they also described ganglion cells which, after rapid initial adaptation,

* The receptive field of a neuron is that area of sensory surface which, when stimulated, can influence the activity of that cell. The receptive field of a ganglion cell in the cat retina varies from approximately 0·5 mm to 3·0 mm[98,99].

showed a maintained discharge, the frequency of which depended upon the level of illumination. One such unit showed a discharge rate of 4 to 8 impulses/sec for a 40-minute period of illumination of 0·3 foot-candles. A tenfold increase of illumination increased the discharge rate to 14 to 17 impulses/sec which remained constant for 35 minutes. If such units, with a non-adapting component of discharge, occur in the human retina, it is difficult to see why a stabilized retinal image should disappear completely. If they exist, the image of the vertical bar is likely to fall across and stimulate a number of them, and this should provide the brain with information about the presence of the bar. In fact, it is highly probable that such cells exist in the human retina, for the pupil, which constricts when light is shone into the eye, remains constricted for long periods of time under conditions of constant illumination. However, the results of experiments with stabilized retinal images seem indisputable, and we must conclude that the information provided by slowly adapting units is not used to resolve detail. It is, therefore, of some interest to find that cats, after bilateral removal of the visual cortex, lack all visual orientating and avoiding reactions, but retain some capacity for brightness discrimination[104].

Only relatively infrequently, in the normal course of events, do we meet objects that stimulate a narrow line of receptors (the sharp edge of a knife, or the fine markings of a vernier scale) or encounter stimulus background relationships that are sharply drawn, such as a black rock framed against a bright white cloud, or a church clock striking on a quiet afternoon. More usually objects tend to rise up gradually out of their surroundings, and this may happen so unobtrusively that their outlines are blurred and the stimulus difficult to discriminate. This difficulty is turned to advantage in the camouflage of many animals whose colouring is so related to their natural background as to make them almost invisible. That the discrimination is not more difficult, seems to be due to the operation of a neural mechanism which serves to improve contrast between stimulus and background.

The first intimation of the existence of such a mechanism in a sensory system came from the work of Galambos[105] on the auditory pathways of the cat, but was first systematically investigated by Hartline[106] on the lateral eye of *Limulus*. It was found[49,106] that a small spot of light shone onto an ommatidium evoked a discharge of impulses in a single fibre of the optic nerve. A few seconds later, while this light remained switched on, neighbouring ommatidia were illuminated and the frequency of spike discharge elicited by the spot was diminished (*Figure* 6.5(*a*), upper trace). In other experiments it was found that nearby ommatidia often inhibited each other mutually. In one of these experiments records were taken simultaneously from two optic nerve fibres, whose ommitadia were 0·7 mm apart. A spot of light was focused on one ommatidium, and while this light remained on, the second ommatidium was illuminated, initiating a train of impulses in its fibre, and at the same time slowing the discharge from the first receptor unit (*Figure* 6.5(*a*), middle trace). The roles of the two ommatidia were then interchanged, and the discharge from the second ommatidium was inhibited by illumination of the first (*Figure* 6.5(*a*), lower trace).

Evidence for the existence of similar inhibitory interactions in the vertebrate retina has now emerged and is also very strong. Barlow[107] recorded

the discharge of single ganglion cells in the frog retina, while exploring their receptive fields with a small spot of light. He confirmed and extended the earlier observations of Hartline[102] that the receptive field of a single cell is roughly circular in shape, with a diameter of approximately 1 mm, and that the field is not uniformly sensitive to light. A spot of light focused on the centre of the receptive field evoked a brisker discharge of impulses than one shone on the peripheral part of the field. In Barlow's[107] experiments the diameter of the spot could be varied, and the intensity of light required to

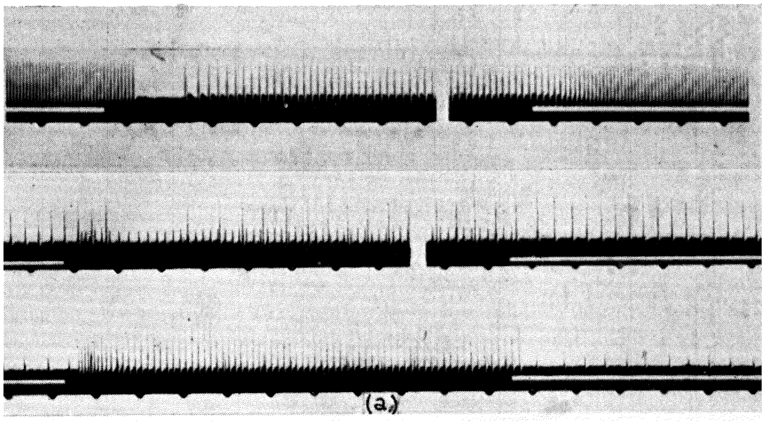
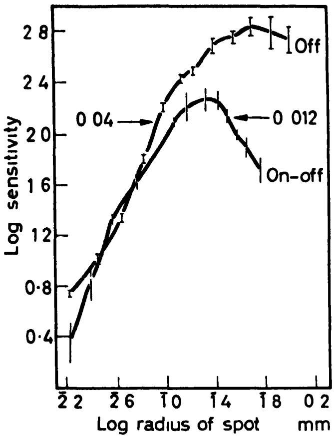


Figure 6.5(a). Effects of lateral inhibition on the discharge of single fibres in the optic nerve of the compound eye of *Limulus*[49]

Upper record: A spot of light was focused on the ommatidium in which the nerve fibre originated. During the interval indicated by a blackening of the white line above the time marks, another beam of light was focused on an annular region surrounding this ommatidium, and the discharge in its optic nerve fibre was inhibited. *Middle and lower records* are from a strand of optic nerve containing two active fibres, whose responses may be distinguished by the different sizes of their action potentials. The two fibres mutually inhibited each other. Each ommatidium could be independently illuminated. The discharge of the fibre (middle record), which gave the large spikes during steady illumination of its ommatidium, was slowed when a light, shone on the second ommatidium, excited the fibre which generated the small spikes. When the roles of the two units were interchanged (lower record), the frequency of the small spike was decreased during the discharge of the large one. (Interval between time marks is 0·2 sec).

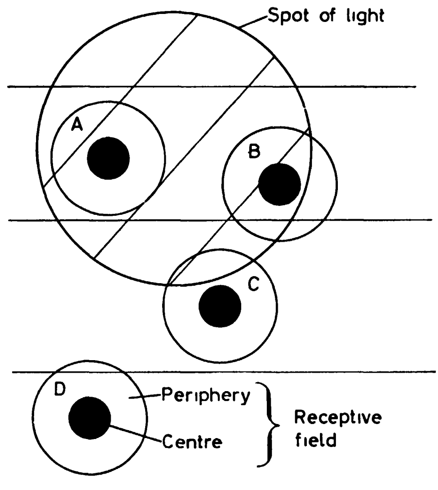
give a detectable (threshold) response in a ganglion cell was measured. The logarithm of the reciprocal of this value gave the logarithm of the sensitivity of the unit. Sensitivity was then plotted against the radius of the spot of light (*Figure 6.5(b)*). Logarithmic scales were used because of the wide range of values covered. The curve of the 'off' unit, one which gives a burst of impulses when a stimulating spot of light is extinguished, shows that the intensity of light required to give a threshold response decreased approximately linearly as the radius of the spot was increased up to a maximum of about 0·1 mm. Within the area described by this radius the excitatory effects generated by light were summated by the ganglion cell. When the radius of the spot exceeds 0·2 mm the curve begins to flatten out. This

suggests that the more peripheral parts of the receptive field add progressively less to the excitatory activity impinging on the ganglion cell. The behaviour of the 'on–off' units, ganglion cells which give a brisk discharge of impulses when a light is switched on and when it is switched off, is dramatically different. The radius sensitivity curve of one of these cells is given in *Figure 6.5(b)*. When the radius exceeds approximately 0·25 mm the sensitivity of the unit drops precipitously. This behaviour strongly suggests that the peripheral part of the receptive field actively inhibits the ganglion cell. This hypothesis receives support from the observation that impulse activity



(b)

(*b*) Radius sensitivity relations in an 'off' and an 'on–off' ganglion cell of the frog retina[107].



(c)

(*c*) Diagrammatic representation of a small area of a cat's retina to illustrate the effects of lateral inhibition on the discharge of ganglion cells. The retina is assumed to be diffusely illuminated, and on it is focused a spot of light.

generated in a ganglion cell by a spot of light falling in the centre of its receptive field, is inhibited by a second spot 0·5 mm away. Substantially similar observations were made by Kuffler[98] in the light-adapted retina of the cat, although here the antagonism between central and peripheral regions of the receptive fields appears to be even more complete. A ganglion cell that gives a burst of impulses when light, focused on the centre of its receptive field, is turned on (on-centre unit) may, when the spot is shifted 0·6 mm, have this discharge completely suppressed. In addition, an 'off' discharge may appear when the peripherally placed stimulus is extinguished. When both spots of light were presented simultaneously, the resulting discharge depended on the intensity relationship of the two. If the central stimulus was relatively stronger, then both stimuli would evoke an 'on' discharge only. If the peripheral stimulus was relatively stronger, the 'on'

discharge would be suppressed and only an 'off' discharge elicited. When both test flashes were adjusted to give an approximately equal discharge, then their interaction resulted in mutual suppression, giving a relatively weak 'on–off' response.

In general, it was found that the response evoked by stimulation of the central zone predominated over that of the periphery. Area threshold experiments gave similar results to those found in the frog retina, namely, that as the diameter of the spot was increased to enclose more of the peripheral region, there was an increase in threshold of the central response[108]. The diameter of the central summating region of the receptive field was variable, the smallest being found near the area centralis in the cat[108], and near the fovea in the monkey (20 μ)[109]. Wiesel[108] found that ganglion cells with small central regions tended to have large, strongly antagonistic peripheral zones, and those with large centres to have relatively narrow and less influential surrounds. This mutual antagonism between centre and periphery is known as lateral or spatial inhibition.

The way in which lateral inhibition might be expected to improve contrast between object and background may be illustrated in the following way. Let us suppose that a spot of light is focused on the retina which is already illuminated by a diffuse and weaker light. Receptive fields in positions A and D in *Figure 6.5(c)* will be completely covered with light, and generate only a weak discharge in the ganglion cells with which they are connected, because both central and peripheral regions of the fields are being stimulated. Since the light falling on field A is more intense than that falling on D, it is possible that the ganglion cell of field A will discharge at a faster rate than that of D. The fields of other cells will lie in relation to the edge of the spot, and are represented by B and C, although, of course, there will be fields lying in intermediate positions. Ganglion cells with fields in position B will tend to give a strong response, of the type characteristic of its centre, since much of the relatively insensitive periphery lies in the less intensely illuminated region. A cell with its field in position C may well be 'silent', or show only weak activity. This is because its central portion lies in the dimly illuminated region, while part of the antagonistic periphery lies beneath the bright spot of light. In summary, cells with fields in position B will show the highest discharge rate and cells with fields in position C will probably show the lowest discharge rate. That is, there will be a region surrounding the spot from which impulse activity is actually lower than that in fibres whose fields lie in the area of background illumination. This trough of neural activity is unlikely to be affected by eye movements since the fast oscillatory movements (approximately 100/sec) which occur between the large, relatively infrequent, saccadic movements (approximately 1/sec) will shift the image, on average, rather less than the diameter of a cone, insufficient[100], therefore, to generate 'on–off' responses in the ganglion cells. The effect of the trough will be to make the discharge of cells with type B fields stand out very sharply. This sharpening up, or improvement in signal to noise ratio at the edges, will be supplemented by the relatively weak activity of cells with type A fields, and will serve to emphasize the boundaries between regions of different brightness, facilitating discrimination of contours. In the absence of lateral inhibition, with activity in the whole

of each receptive field summating at the ganglion cell, the frequency of discharge of the cells (designated by their respective fields) would be arranged in the following order A > B > C > D, and boundary lines would tend to be smoothed instead of sharpened.

In the above paragraph, the activity of ganglion cells with 'on' centres and 'off' surrounds was discussed. We might easily have chosen to discuss ganglion cells with 'off' centres and 'on' surrounds, in which case the pattern of discharge along the optic nerve would have been similar, but in the opposite direction to that described above. For example, the cell of field B, instead of being the most active of the group, would become the least active, and the cell of field C, instead of being the least active, would beome the most active. The image sharpening effects of lateral inhibition would thus be retained.

The expectation that regions of brightness contrast evoke greater neural activity than regions of uniform illumination is confirmed by the work of Grafstein, Burns and Heron[110]. These workers used curarized cats whose brains had been transected in the region of the inferior colliculus. Curare would probably abolish any eye movements that the cat might otherwise exhibit. A rectangle of light was flashed on to the retina and the evoked discharge recorded in a single unit at the visual cortex. The stimulus was then shifted so that a different part of it fell on the retinal receptive field of the cell, any change in the discharge pattern of which was recorded. When a wide bar was used as stimulus, the unit showed two peaks of activity, one when each of the edges of the bar lay on its retinal field. Among the neurons studied, whose activity was markedly influenced by the position of the stimulus, about 90 per cent were found to be edge-sensitive. The remainder showed only a poorly defined peak of activity corresponding to the centre of the bar. Clearly, with this arrangement, there is some loss of information about the absolute level of illumination of images, but this is offset by the accentuation of brightness differences.

The organization of receptive fields into regions mutually antagonistic to each other is almost certainly connected with the discrimination of the more complete aspects of an image that occurs in the cerebral cortex. Hubel and Wiesel[111] found that some of the retinal receptive fields of single units in the visual cortex of the anaesthetized cat were cigar shaped, with a central zone flanked by antagonistic areas. The long axis of each of these fields was oriented in any direction on the retinal surface. The flanks were often asymmetrically distributed about the centre. The effect of movement on the activity of a cortical unit was investigated by moving a slit-shaped bar of light across its receptive field. When a horizontal slit was moved up and down over a vertically orientated field, no response was elicited in the cell, presumably because the slit lay across both excitatory and inhibitory areas, and the mutual antagonistic effects of each was complete. When a vertical bar of light was moved back and forth across the same receptive field, a response was elicited at each crossing. In this case the stimulus moved across the antagonistic parts of the receptive fields successively and each part could exert its effect unopposed. In some units the response to crossing in one direction was brisker than that to crossing in the opposite direction, and this effect could sometimes be accounted for in terms of the organization

of the receptive field. The receptive field of one such direction-sensitive unit consisted of a strong inhibitory zone flanked by two excitatory areas, of which the right was weaker that the left. A slit of light, with its long axis parallel to that of the field, produced a strong response when moved to the left, but only a feeble response when moved to the right. The best response, therefore, was obtained in moving from the inhibitory to the stronger of the two excitatory regions. Units of this sort would obviously provide information about the course taken by an image moving across the retina. Furthermore the image of a moving object may be expected to evoke greater activity in the visual pathways than a stationary one since it presents a continuously changing front of retinal excitation. These differences provide a plausible explanation of the fact that a moving object is more easily detected than a stationary one.

Lateral inhibition is not restricted to the visual pathway, but occurs in other sensory systems. Mountcastle[112], recording from cells in the somatic sensory cortex of the cat, was able to excite them by deforming hairs lying in a localized area of the skin. One such unit, which had an excitatory receptive field approximately 1·5 cm in diameter, could be inhibited by stimulation of an extensive area, concentric with its excitatory field. Similar observations have been made on single neurons in the somatic sensory cortex of the monkey[113,114], where stimulation of the inhibitory region of the cutaneous receptive field suppressed both spontaneous and evoked activity. There is good reason to suppose that in the auditory system, too, inhibitory interaction occurs between functionally related neurons. Galambos[105] found in the auditory pathways of the cat, that the response evoked in a neuron by one tone could be inhibited by the simultaneous sounding of another. Occasionally, conditions were found where either tone could stimulate when sounded alone, but the addition of the second inhibited the response. These results suggest that mutual inhibition will occur between fibres at the edge of overlapping arrays of active neurons. When two such arrays are conducting impulses, units in the region of overlap will be inhibited, so that between the two groups of fibres will be a region of relative quiescence. The two zones of activity remain distinct, and their boundaries are therefore sharpened[115].

Mutual inhibitory interaction between neurons in the afferent pathways ensure that the brain is provided with a maximum of information about regions of the environment in which spatial configurations change, and a minimum of information about regions in which the spatial conditions are held constant. These effects may be compared with those resulting from adaptive processes occurring in sense receptors, where a stimulus which changes in time evokes greater activity than one which does not. Both processes lead to the economic use of impulses in the transmission of information about stationary conditions in the environment, and to maximum discrimination with respect to conditions that are not constant.

Very little is known about the mechanisms which mediate inhibitory interactions between sensory neurons. Hartline and his colleagues[49] have shown that in the compound eye of *Limulus* the interaction depends on the integrity of neural connexions within a plexus of nerve fibres that lies behind

the layer of ommatidia. In many of their experiments, a single fibre was dissected from the optic nerve and isolated up to the ommatidium from which it originated. During the course of such dissection, the inhibitory effect diminished progressively as the fibre bundle was cut away from its connexions within the plexus. When the dissection had been extended up to the pigmented body of the ommatidium, no inhibition could ever be obtained by illuminating adjacent regions of the eye. We may take it that the fibre continued to discharge in the normal way when its own ommatidium was illuminated and, therefore, that failure to obtain inhibitory interaction was probably not due to damage to that fibre sustained in the course of dissection, although this point is nowhere made explicit in the paper[49]. If, on the basis of these experiments, we draw the unwarranted, but attractive conclusion that all inhibitory interactions are dependent on the integrity of neural interconnexions, it is tempting to take the further step and suppose that these interactions are mediated by a mechanism of the Renshaw type (p. 248 *et seq.*) that is, a discharging neuron sets up activity in another whose axon terminals release an inhibitory transmitter substance which reduces activity in other cells. In its simplest form, however, this hypothesis is not adequate to account for all the characteristics of mutual inhibition as seen, for example, in the visual system. There are no nerve cell bodies in the sub-ommatidial plexus of *Limulus*[49], so that if recurrent collaterals from an active optic nerve fibre inhibit activity in an adjacent fibre, they do so in the absence of an interneuron.

It is more difficult to explain the results of Barlow, *et al.*[99] on the cat retina in this way. They found that the sort of response that can be elicited in a ganglion cell by light falling on its receptive field varies with the level of background illumination. A ganglion cell which in the light-adapted eye gives a duplex response, with an 'on' centre and an 'off' periphery, comes to have only 'on' properties after the eye has been dark-adapted. That is, the antagonistic effect of the periphery of a receptive field in the light-adapted retina, disappears after a prolonged period in the dark. Under these latter conditions, therefore, the pathways mediating lateral inhibition are not active. However, ganglion cells often are[103] and so, presumably, would be the hypothetical inhibitory interneurons which, in the Renshaw type of system, are driven by collaterals of the active cell. Brown and Wiesel[67] thought that the receptive fields of bipolar cells had similar antagonistic central and peripheral regions[55], so it is possible that the antagonistic effects are mediated by inhibitory interneurons driven by these cells. This is unlikely because, like ganglion cells, bipolar cells show impulse activity in total darkness. It seems probable, therefore, that the mechanism that mediates lateral inhibition in the retina differs in some way from that which mediates inhibitory interactions between anterior horn cells in the spinal cord. Nevertheless, although these two processes do not seem to be identical, there are striking analogies between them. A clearer understanding of the mechanism of lateral inhibition and its relationship to recurrent inhibition, may well emerge when more is known of the functional connexions between photoreceptors and bipolar cells, and of the significance of the connexions between adjacent photoreceptors which have been described by Sjostrand[116] in the eye of the guinea-pig.

THE CONTROL OF TRANSMISSION

The period of depressed excitability that follows the action potential in a nerve cell is usually much longer than, and follows, the period of increased excitability (p. 245). Therefore a stimulus which is sufficiently intense to generate an impulse in such a cell, also generates in that cell a process which tends to choke off any further response to the stimulus. A similar effect may be brought about by recurrent inhibition. A cell may, through its recurrent collaterals, excite an inhibitory interneuron. If this interneuron feeds back on to the cell that excites it, the discharge in that cell may be slowed down or, possibly, completely suppressed. Both these inhibitory processes are impulse-dependent, and very probably exercise a fixed, and hence relatively inflexible, control over the transmission of impulses. There are, however, some rather more versatile ways in which sensory inflow into the nervous system may be controlled. If we close our eyelids, evoked activity in the optic pathways will be attenuated, and if we lie still and relaxed, impulse activity in neurons connected to muscle and joint sense organs will be greatly reduced. A fly crawling across the back of a dog sets up neural activity which is terminated when the insect has been flicked off (scratch reflex[117]). The control of input exercised in these ways is clearly quite coarse, and the question has been raised, at various times in the past[118–120], whether some other mechanisms might exist through which a more delicate and flexible control might be affected. In the last few years evidence has been accumulating, which is almost compelling in abundance, although variable in quality, which suggests that such mechanisms do exist and that the brain exercises control over the signals delivered to it, by a system of peripherally directed fibres, passing to and modifying the excitability of the sensory pathways.

The earliest and most convincing work in this field was done on the mammalian muscle spindle. The stretch-sensitive region of this receptor is associated with a specialized mass of muscle tissue, the intrafusal fibres, through which the sensitive region may be connected to the ends of the muscle[121]. When the muscle is stretched, a discharge is set up in the afferent fibre from the muscle spindle and is conducted to the spinal cord. If the spindle were slack when a load is applied across the ends of the muscle this slack would have to be taken up before a signal could be generated in the afferent fibre. In fact, the intrafusal fibres receive a motor innervation independent of that to the extrafusal fibres[122]; so the degree of stretch to which the spindle is subjected can be varied independently of the length of the muscle[123]. When the muscle is relaxed, the intrafusal fibres may, through their separate motor nerves, be contracted, and the stretch-sensitive region of the spindle held under slight tension. If, now, the muscle is suddenly stretched, the response of the spindle is almost instantaneous. The sensitivity of the stretch receptor is thus brought under the control of the central nervous system[124] through the small motor fibres that innervate the contractile elements of the receptor itself. Hagbarth and Kerr[125] subsequently showed that the conduction of signals in the sensory pathways might also be subject to control by the brain. The lower half of the spinal cord of anaesthetized cats was exposed and a dorsal root in the lumbar region stimulated by an electric current. Some of the fibres in the dorsal

root enter the cord and synapse with cells in the dorsal columns of grey matter. The axons of these post-synaptic neurons cross the midline and carry impulses to the brain in the ventral columns of white matter. Hagbath and Kerr found that electrical stimulation of various structures in the brain, including the anterior vermis of the cerebellum, the midbrain reticular formation, and the somatic sensorimotor area of the cerebral cortex, resulted in a reduction in amplitude of the dorsal root-evoked volley in the ventral columns. In a later paper, Kerr and Hagbath[126] showed that the amplitude of evoked activity in the olfactory pathways of the curarized cat could also be attenuated by stimulating certain structures in the brain, and Galambos[127] showed that a click-evoked response in the auditory nerve could be depressed by stimulating a restricted region of the brain stem. In all these experiments the potentials recorded were not those of single fibres, but the massed response of many, and in none of the experiments was the amplitude of the evoked response increased by central stimulation.

These results suggest that there is, indeed, a centrifugally directed system of fibres within the nervous system, the function of which is to modulate activity in the sensory pathways, although we might expect to get more detailed and precise information about its function by studying the effects of central stimulation on the discharge of single afferent neurons. Hagbarth and Fex[128] used capillary electrodes to record the activity of such units in the spinal cord of cat. These units were activated from the periphery by, for example, pinching the skin or moving a joint, and according to their response, and on the basis of the presence or absence of spontaneous impulse activity were classified as pre- and post-synaptic. Some units classified as post-synaptic were inhibited, some activated and others uninfluenced by central stimulation, which on no occasion affected the activity of the pre-synaptic units*. Granit[19], also working with the cat, showed that it was possible to alter the spontaneous activity of retinal ganglion cells, and their response to flashes of light, when certain structures in the midbrain were stimulated. Many optic nerve fibres terminate in this region, so there was a very good chance that some of them would be activated by the stimulating current and backfire into the retina. Granit studied these antidromic effects by recording from ganglion cell bodies in the retina while deliberately stimulating their axons as they passed through the superior colliculus in the midbrain. Under these conditions the frequency of discharge of the retinal units was determined by the frequency of the applied electric shocks, that is, the cells were driven by the stimulus. At the end of the period of stimulation, the rate of discharge of some cells was increased and that of others decreased, for periods of up to 20 sec. When the stimulating electrodes were pushed deeper, into the midbrain reticular formation, it was found that the ganglion cells were no longer driven, although there was sometimes a change in the frequency of their spontaneous discharge. After the stimulus had been switched off, an increase was observed in the rate of firing of many of these 'undriven cells'. More rarely, cells showed a period of inhibition following the stimulus. Granit felt that the facilitatory and inhibitory effects elicited without 'driving' were not antidromic but the result of activity in a centrifugally directed system of fibres.

* Anderson and his colleagues[128a] consider that primary sensory neurons in the spinal cord may be effected by central stimulation.

The experiments of Granit and of Hagbarth and Fex cast an interesting light on the significance of the work on massed evoked responses mentioned above. Central stimulation always depressed, never enhanced, the size of these evoked potentials, and it has been concluded that the structures from which these effects can be elicited exercise a purely inhibitory control over the sensory pathways. This conclusion is unwarranted and, in the event, has proved false. Furthermore, a massed evoked response might be reduced when activity in individual fibres in the pathway is actually increased, since its amplitude often depends not only on the magnitude of the stimulus, but on the amount of background activity already going on in the pathway. If the background activity in the optic nerve, for instance, were increased by an increase in the rate of discharge of individual fibres, we might expect the massed response to be 'drowned' in the background noise[130]. It is dangerous to extrapolate to the behaviour of single units from the behaviour of the massed evoked response; it is foolhardy to do so without information about this background noise.

The anatomical and physiological evidence for the existence of a centrifugally directed system of fibres exercising a direct (neural) control over the sensory inflow at the periphery, is not uniformly good. It is best for the proprioceptive system (muscle spindle), non-existent for the taste pathway, equivocal for vision, and a confirmed sceptic might challenge the evidence for its existence in the auditory pathways. The minimum requirements that must be fulfilled to establish the existence of such a system, beyond reasonable doubt, are: (*a*) anatomical evidence of centrifugal fibres, which terminate on neural structures, and which are not recurrent collaterals of the sensory neurons themselves, and (*b*) satisfactory evidence that the effects of central stimulation are orthodromic, not antidromic, and that these effects are not a consequence of changes in blood flow following stimulation of vasomotor fibres. If centrifugal fibres in a given pathway turn out to be vasomotor, then the control such a system could exercise over sensory inflow is likely to be quite gross and relatively non-specific.

If we apply the above criteria to the evidence for the existence of a centrifugally directed system of fibres passing to the mammalian retina, we shall see that the evidence is not unchallengeable. It might seem a simple matter to demonstrate the presence of axons in the optic nerve whose cell bodies lie in the brain-stem, but in practice, this has proved extremely difficult to do. Fibres have been described in the eye of the dog[131] and chimpanzee[132] which appear to break up and terminate in the retina, and it has been suggested that the cell bodies of these fibres may lie outside the retina. Polyak, however, states that when the optic nerve of a monkey is severed, some ganglion cells show no sign of degeneration (see reference 59, p. 281). Such neurons probably lie wholly within the retina and the terminal arborizations of their axons could be mistaken for the endings of centrifugal fibres. Experiments, in which one eye has been removed and evidence sought for fibres in the optic nerve that have not degenerated, have been inconclusive. Optic nerve fibres that persist after enucleation of the corresponding eye[133,134] are not necessarily the axons of neurons with centrally placed bodies. They could be sprouts from fibres in the intact optic tract, induced to develop collaterals by the process of degeneration in those fibres

affected by enucleation. Hess[135] found complete degeneration of fibres in the optic nerve of foetal guinea-pigs 4 days after the removal of the eye. The close correlation between the number of ganglion cell bodies in the retina and the number of axons in the optic nerve[33,136] provides additional evidence against that view that centrifugal fibres exist, in any great number, in the mammalian optic nerve.

So far as the physiological evidence[129] is concerned, it is possible for retinal ganglion cells to be activated from the midbrain in at least two ways, neither of which require the existence of centrifugal fibres: (*a*) by antidromic activation of the cell being monitored, or (*b*) by antidromic activation of cells adjacent to the one being monitored. It seems probable that the former is the means by which 'driving' takes place, but some cells, driven in this way, may be so small as to escape detection by the relatively large recording electrodes (25 μ tip diameter) which Granit used. Such cells could influence the activity of adjacent neurons, either through recurrent collaterals, or through interneurons (*e.g.* centrifugal bipolars[59]). Adjacent ganglion cells activated trans-synaptically in this way, may well not be driven by the central stimulus, and hence fall into the class of units which Granit, possibly mistakenly, considered were activated by a centrifugal system of fibres. With regard to this interpretation of events, the work of Phillips[137] is particularly interesting. He recorded from single neurons (Betz cells) in the motor cortex of the anaesthetized cat, while electrically stimulating their axons in the pyramidal tract. Betz cells, which at certain shock strengths could be driven anti-dromically by pyramidal stimulation, were not driven by weaker stimuli. These weak shocks, however, caused excitability changes in the recorded cell. Phillips considered that under conditions when a cell was not driven antidromically, changes in its excitability were brought about through impulse activity in recurrent collaterals of adjacent, antidromically activated Betz cells. Finally, as Granit points out, some of the effects of central stimulation could be secondary to alterations in blood flow through the retina. Such changes have been observed following stimulation of the brain-stem reticular formation (Ingvar, quoted by Granit[129]).

The sheer bulk of work that has been done in the past six years or so on the problem of centrifugal control of the sensory inflow, is awe-inspiring[138], and of this Granit's work[129] on the retina is among the most rigorous. But where, and for so long as, criticisms of the sort mentioned above, have to be admitted, the hypothesis that the input to a given sensory pathway can be controlled directly at the receptor level, or at the first central synapse, through a centrifugally directed system of neurons, is weakened. Before this hypothesis becomes too firmly entrenched on our way of thinking about the mammalian central nervous system, it is well to remember that a good deal more experimental work will have to be done before it is established beyond reasonable doubt for all the sensory pathways. This is not to say that the evidence for the existence of such a centrifugal controlling system is weak for every pathway, but that in some, at least, it could be stronger.

Setting aside the question of where this control is exercised, there is good reason to suppose that sensory signals on their way to, or at, the cerebral cortex undergo some sort of editing, since their passage can be influenced

by the animal's behavioural state and by activity in other sensory pathways[130,139,140]. Some of the experiments on which these observations are based have been performed on unanaesthetized animals which were free to move within their training box. In one of these experiments[130] a stroboscope was placed in front of the box and the response to a flash of light recorded through electrodes implanted on the visual cortex. After the cat had become adapted to its surroundings, and was relaxed, it was allowed to watch a mouse running on a ledge in front of the training box. The cat at once showed interest in the mouse and during this period the size of the massed evoked response to light was reduced compared with its amplitude in the period before the mouse was introduced. These changes in size of the evoked response appeared to occur independently of changes in the background electrical activity of the brain, and were probably not secondary to autonomic effects on the cerebral circulation. When the cat showed no behavioural evidence of interest in the mouse, or when a tone was sounded and the cat's interest appeared to be abstracted from the field of vision, the amplitude of the evoked response to light was at its maximum.

These results indicate that the total current generated in the production of a photically evoked response is reduced when an animal's behaviour is directed to events in the fields of vision, but is not reduced when its interest appears to be abstracted from vision. It is difficult to appreciate the significance of these results until more is known of the relationship between the massed evoked response at the cortex, and the elements which generate it. It is known[141,142] that certain components of the response at the visual cortex are related to activity in thalamocortical fibres, and assumed[143] that other components are generated by neurons in the cerebral cortex, although shifts in membrane potential of glial elements[144] may contribute and have not been excluded. As a first approximation, however, the results suggest that signals reach and elicit activity in, the cerebral cortex of an 'unattended' modality, for the evoked response to light remained the same whether the flash was delivered alone or preceded by a distracting sound. Even if we assume that the massed evoked response at the cortex is generated exclusively by the activity of large numbers of neuronal elements, we cannot argue that because the size and shape of the response remain the same in different experimental situations, the *distribution* of activity among these elements is unchanged. Indeed, it is theoretically possible for substantial changes to occur in this distribution, from one experimental circumstance to another, and yet for the dimensions of the evoked response to remain the same.

Some evidence in support of this view comes from experiments[145] which are still in progress*. Like the experiments described above[130], they are designed to investigate the effects of a competing sensory stimulus on the cortical response to a flash of light in the unanaesthetized cat; unlike the earlier study, the discharge of single units is being examined. Flashes of light are delivered from small neon bulbs mounted on contact lenses which the animal has been trained to wear. Since the pupils are dilated, and the nictitating membrane removed at a previous operation, the amount of light falling on the retina is constant. A microelectrode, mounted on a small, moving platform (*Figure 6.6(a) and (b)*) is gently lowered into the cortex[145a],

* Supported by a grant from the Medical Research Council.

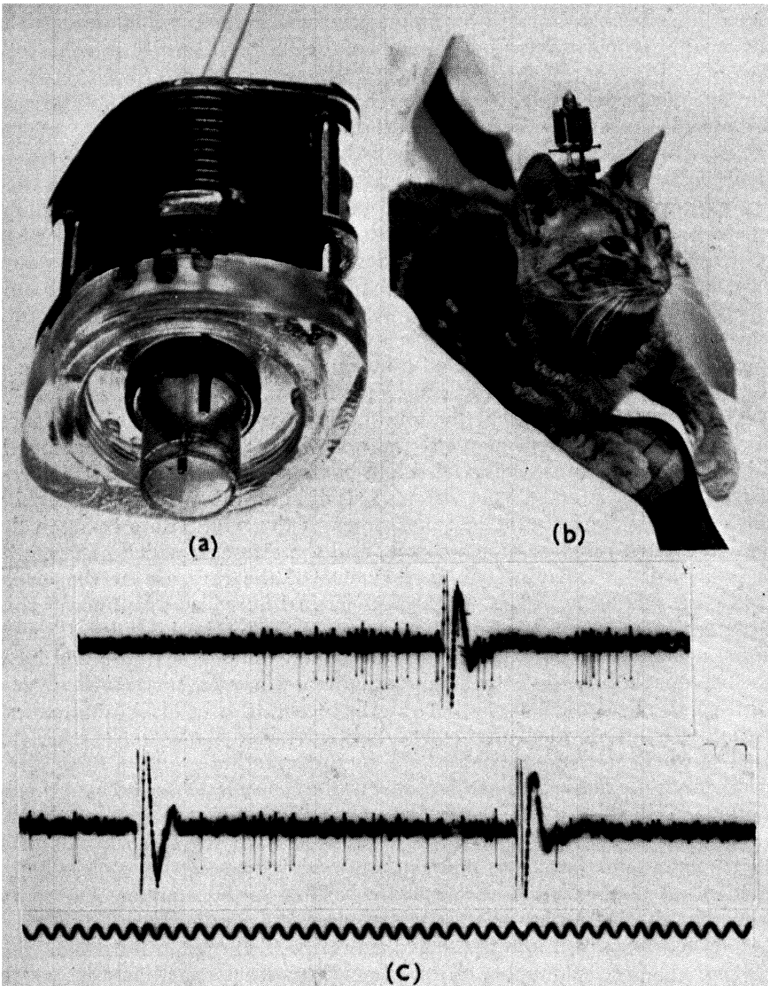


Figure 6.6. (*a*) Instrument used for recording single units from the cerebral cortex of the unanaesthetized cat. Each steel tube holds a tungsten microelectrode. Signals conducted by the electrode are passed through the tube to the screw, amplified and stored on magnetic tape. The bellows are expanded, and electrodes lowered into the cortex through a hole in the skull, by the passage into the bellows of liquid paraffin through the Polythene tubing. (*b*) Instrument in position on cat's head. (*c*) Unit which responds to retinal illumination, a flash of 4 msec duration recorded from visual cortex of an unanaesthetized cat. The response (upper record) to a flash (stimulus indicated by artefact) is substantially reduced (lower record) when the flash is preceded by a shock (first artefact). The time mark, a 50 c/s ripple, is the same as for both records.

which is explored for units which respond to light. When such a unit has been found, a series of flashes is given, and this is followed by a series in which each flash is preceded by a skin stimulus. In one unit the number of spikes elicited by a flash when it was preceded by a shock, was greater, and in another less (*Figure 6.6(c)*) than when the flash was delivered alone. In another unit the total number of spikes was increased, but the latency of the discharge, the time that elapsed between delivery of the flash and the first spike of the response was also increased. Clearly a complex reorganization of cortical activity may occur in circumstances in which the evoked response may be expected to be unchanged.

Reduction in amplitude of the cortical evoked response to a flash of light, as when an animal appears to be attending to information conducted over the visual pathways[130], may result from a reduced input to the cortex, and/or from a reduced responsiveness of the cortex to the incoming volley, or may, and probably does, result from a reorganization of signals[128] in the sensory pathways. If this reorganization leads to a reduction, on average, of impulse traffic in these pathways, it does not necessarily follow that the channels are functioning less efficiently, or that their threshold to the type of stimulus evoking the impulses is greater. It may be that inhibition accentuates differences and leads to a sharpening of image contours.

To conclude, it is certain that sensory pathways are not, as was once thought, uncontrolled, passive channels for conducting messages from the sense receptors to the brain. Signals passing along these channels may be reorganized in a way that is not yet clearly understood, but the changes may often be correlated with changes in the animal's orientation to the stimulus which evoked the signals.

EVALUATION OF SENSORY INFORMATION

It seems reasonable to suppose that the significance of a particular sensory input, and our evaluation or interpretation of it, will depend on whether or not we have experienced something like it before, and on the information arriving along other sensory pathways. The importance we attribute to a loud bang, for example, will depend on the circumstances in which we hear it. If we heard it on 5th November and a moment previously had seen a flash of light from the same direction, we would probably conclude that the bang was caused by a firework exploding. If we were subject to the same sensory experience, but happened to be on a battlefield, we might evaluate the information differently and respond in a different way.

In order to understand this process of evaluation of sensory messages, we need to know how and where information is stored in the brain, and how the mixing of intercurrent and past information takes place. With regard to the former (the question of how information is stored), theories abound, but sound experimental data are almost entirely lacking. One attractive hypothesis is due to Hebb[146], later modified by Milner[147]. It is suggested that when signals enter the brain, they generate activity in groups of neurons which, through repeated stimulation, gradually come to fire together. This group of neurons, known as a cell assembly, is considered to constitute the simplest instance of a representative process, and will discharge, for instance,

when an appropriate pattern of impulses arrives to trigger it off. The hypothesis assumes that when cells have been activated together, the probability of their discharging together in the future, is increased. This functional coupling is brought about by a growth process which, Hebb suggests, accompanies synaptic activity and makes the synapse more readily traversed. There is a great deal of evidence (recently reviewed by Hughes[148]) to show that the effectiveness of a pre-synaptic volley in eliciting a spike in the post-synaptic neuron, is greatly enhanced for a period of minutes or hours, after an intense burst of action potentials in the pre-synaptic fibre. Since the required frequency of this discharge is well within the physiological range, this phenomenon, which is known as post-tetanic potentiation, may well occur in the intact animal. Memories, and hence the linkage between neurons in an assembly, often last longer than a few minutes or hours, so that post-tetanic potentiation cannot, in itself, be the basis of these presumed couplings. It is possible, however, that permanent organic changes take place during the course of post-tetanic potentiation. One such change would be a growth in size of the synaptic knobs which, when active, would depolarize a larger area of membrane and be more likely to generate a spike in the post-synaptic neuron. So far as the author has been able to find out, however, no such growth changes have ever been observed. An alternative site for the hypothetical changes by which neurons may be functionally associated is the post-synaptic membrane. It is quite conceivable[119] that changes on this side of the synapse may affect transmission if some quite plausible assumptions are made. If the number of molecules of transmitter substance liberated by synaptic knobs exceeds the number of reactive groups with which the molecules combine at the post-synaptic membrane, an increase in the number of reactive groups would lead to increased efficiency of synaptic transmission. Since the receptor molecule is probably a protein or lipoprotein, its synthesis is likely to be controlled by ribonucleic acid (RNA) in the cell cytoplasm. Large quantities of transmitter substance, liberated as a result of intense impulse activity in the pre-synaptic fibres, may influence the synthesis of receptor molecules through a primary effect on RNA. Attardi[149] has shown, in rats, that prolonged walking activity which, he considered, caused intense stimulation of cells in the cerebellar cortex, was associated with an increased concentration of RNA in the cytoplasm of these cells. These findings are consistent with those of Hyden[150] who observed an increased concentration of cytoplasmic RNA in neurons of the vestibular pathways of rabbits which had been subjected to intense rotatory stimulation. This and other work on factors influencing the RNA content of neurons in the nervous system are discussed in a review by Morrell[151].

Lashley[152] spent much of his working life searching for so-called memory traces in mammals, but, hypotheses apart, we still know little about the neural correlates of information storage. All that can be said, with any sense of security, is that information storage in mammals, though not, perhaps, in molluscs[153], is not the prerogative of any localized mass of brain cells[154], and that it may be related to the density of neural interconnexions[155,156]. In the course of work on the visual cortex (see p. 273) however, a unit was found which showed some of the characteristics of one

which was part of a short term storage system. This unit is illustrated in *Figure 6.7.* A sequence of flashes was delivered to the cat at the rate of one every two seconds and the response of the unit recorded. After each flash

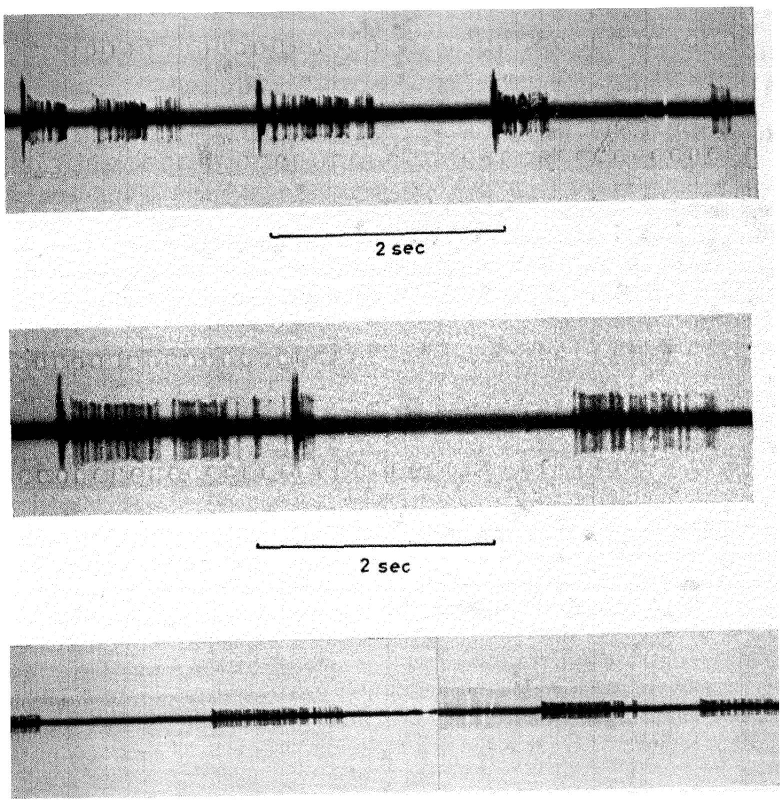


Figure 6.7. A single unit which responds to diffuse retinal illumination (upper record) and to weak electric shocks applied to the skin over the back (middle record). The cat from which these records were taken was free to move about within its training box. *Upper record* shows response to last three flashes (indicated by artefacts) in the series together with a burst which appeared at the time the next flash *would* have occurred, had the series been continued. A similar discharge (*middle record*) occurred at the end of the sequence of skin stimuli, the last two of which are indicated by the artefacts, and thereafter (*lower record*) the unit, no longer driven by an external stimulus, continued to discharge spontaneously at irregular intervals. The last burst of the middle record is the first burst of the lower.

a burst of impulses occurred, which varied slightly in latency and number of spikes. At the end of the series of flashes, the unit fired at the time the next flash *would* have occurred, had the series been continued (*Figure 6.7*, upper record). This unit also responded to a weak electric shock applied

to the skin of the back. At the end of a regular series (one shock every two seconds) of such stimuli, the unit once again discharged when the next stimulus would have occurred had the series been continued (*Figure 6.7*, middle record). Thereafter the unit fired spontaneously in bursts, but the intervals between bursts were long and irregular (*Figure 6.7*, lower record), so it is unlikely that the flashes and shocks were coinciding with this spontaneous discharge. A large number of units such as this will have to be studied before any progress can be made in understanding the presumed short-term storage system of which such units may be part.

Recent studies of memory in human subjects[157–159] have yielded some extremely interesting results. It was found that bilateral lesions of the hippocampus and hippocampal gyrus are followed by a severe and generalized loss of recent memory. Psychological studies of these patients[158,159] showed that, in spite of this deficiency, memory of the distant past was intact, and that there was no defect of attention, concentration, reasoning ability or previously acquired skills. Milner[160] concluded from these studies that the hippocampus and hippocampal gyrus play a crucial role in the retention of new experiences.

Because of the paucity of information about the neural basis of memory, we cannot say how intercurrent and past information are related in the process of evaluation of a given sensory input. However, a great deal has been learned in the past decade or so about the function of a certain region of the brain and this work may cast some light on the problem. The region is known as the brainstem reticular formation and lies between the long ascending sensory pathways that pass to the thalamus. The reticular formation receives fibres from these pathways, from the cerebellum, and and from the cerebral cortex[161]; fibres pass from it to the spinal cord, the cerebellum, and also, probably, to the thalamus and hypothalamus. Electrical stimulation of the reticular formation can influence the discharge from muscle spindles[124] and induce widespread, bilateral changes in the electroencephalogram recorded from the cerebral cortex of the cat[102]. Furthermore, small lesions in this area lead, in man, to a profound loss of consciousness, whereas damage to a relatively large area of cerebral cortex may be sustained and consciousness remain unclouded[163]. Lesions placed bilaterally in the midbrain reticular formation of the cat[164,165] are followed by a prolonged state of coma, although lesions in the afferent pathways only, beyond the level at which their axon collaterals pass into the reticular formation, are not. This region, with its extensive connexions, and with its tremendous potential for controlling activity in widespread regions of the central nervous system, is bound to play a highly significant part in sensory integration, though how it may do so is still largely a matter of speculation.

CONCLUSIONS

The work that has been done on the nervous system over the past 25 years has necessitated a fundamental revision of the views concerning its operation. It is no longer adequate to think of the sensory pathways as a system which passively conducts a coded, although faithful, representation of images thrown on the receptive surface, but as a system of communication channels

in which complex operations are performed on the sensory input. Although much has been learned about the coding of information in the central nervous system through the use of anaesthetized animals, there is a limit to what can be learned from these preparations, for it is certain that anaesthetics alter the properties of the nervous system, often in a profound way. Work with such animals, therefore, will need to be supplemented by work with conscious, 'behaving' animals if we are to understand more fully the neural mechanisms of sensory integration. Such studies, and particularly electrophysiological investigations of the discharge patterns of single neurons, have only recently been undertaken, but it is becoming increasingly obvious as a result of this work, that many of our preconceived notions about the neurological basis of attention, perception and learning, will need to undergo a radical revision.

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INDEX